HIV-1 TAT1-86 INDUCED EFFECTS ON EXTINCTION AND RELAPSE IN FEAR CONDITIONING LEARNING

Ian Randolph Jacobs

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Psychology and Neuroscience (Behavioral and Integrative Neuroscience) in the College of Arts and Sciences.

Chapel Hill
2022

Approved by:

Sylvia Fitting

Donald T. Lysle

Kathryn J. Reissner

Regina M. Carelli

Neil W. Mulligan

ABSTRACT

Ian Randolph Jacobs: HIV-1 Tat1-86 induced effects on extinction and relapse in fear conditioning learning
(Under the direction of Sylvia Fitting)

HIV-1 Associated Neurocognitive Disorder (HAND) is a neurodegenerative condition affecting roughly 30-50% of HIV-1 infected individuals. Symptoms include a wide range of cognitive impairments, but this series of experiments focuses on deficits in learning and memory. Specifically, these experiments investigate behavioral deficits in associative learning using fear conditioning methodology. The transactivator of transcription (Tat), In humans, the severity of symptoms is most strongly correlated with the severity of synaptic disruption and dendritic injury. Of the HIV-1 viral proteins, Tat plays a key role in facilitating structural and functional dendritic defects in neurons. Within the brain, Tat has several direct and indirect effects that result in structural and functional changes to regions of the brain crucial for associative learning. Fear conditioning using a Tat transgenic mouse model of HAND allows for the study of these affected regions while minimizing the influence of motivational systems. In fear conditioning, subjects are presented with a conditioned stimulus (CS) followed by unconditioned stimulus (US). The subject learns an association between the two, and performs a conditioned response (CR), indicative of learning, in preparation for the US. The acquisition of this response can then undergo extinction in which the US is presented multiple times without the US, thus reducing the conditioned response. Finally, relapse in conditioned responding can be observed due to changes in context, and reminders of the US. There are region

specific contributions to each of these learning processes, allowing for the connection between specific deficits and associated brain regions. The current series of experiments investigated acquisition, extinction, and relapse of conditioned responding.

Results from these experiments reveal transient deficits in acquisition for male and female Tat(+) subjects and a transient deficit in extinction learning seen only in male Tat(+) subjects. These findings indicate disruption to amygdala and prefrontal cortex circuitry. Renewal, a contextual form of relapse, was not observed while reinstatement, a US reminder form of relapse, was. These results indicate that the failure to observe renewal were not due to failure to recall acquisition memories. Overall, these experiments establish clear methodology for investigating associative learning deficits in the Tat transgenic mouse model of HAND and demonstrate transient deficits related to acquisition and extinction.

To all the mentors and teachers who have energized my scientific endeavors and without whom I would have never discovered my passion.

ACKNOWLEDGMENTS

I would like to thank all of the undergraduate research assistants that made this work possible with special thanks to James Betts and David Zak for their role in data analysis and coding. I would also like to thank the animal care and veterinary staff for their role in maintaining the welfare of our animals through the studies. Finally, my fantastic mentor and colleagues, your efforts and support were inspirational throughout this project. We gratefully acknowledge the support from the National Institute on Drug Abuse (NIDA R21 DA041903, R01 DA045596, UNC CFAR P30 AI50410, T32 DA007244, R01 DA032933, R01 DA039942, K05 DA021696, and R21 AG042745).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AMG	amygdala
ANI	asymptomatic neurocognitive impairments
ANOVA	analysis of variances
BLA	basolateral amygdala
cART	combined antiretroviral therapies
CEA	central nucleus of the amygdala
CNS	central nervous system
CR	conditioned response
CS	conditioned stimulus
D1	dopamine type 1
Dox	doxycycline
GABAAR α1	gamma-aminobutyric acid receptor subunit alpha-1
GAD 67	glutamic acid decarboxylase 67
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorder
HIV	human immunodeficiency virus type 1
HPA	hypothalamic-pituitary-adrenal

HPC	hippocampus
IACUC	Institutional Animal Care and Use Committee
IL	infralimbic cortex
ISI	inter-stimulus interval
LTP	long-term potentiation
M	mean
MND	mild neurocognitive deficits
NMDA	N-Methyl-D-aspartate
NS	neutral stimulus
PFC	prefrontal cortex
PL	prelimbic cortex
PSD-95	postsynaptic density 95
SEM	standard error of the mean
sEPSC	spontaneous excitatory postsynaptic current
sIPSC	spontaneous inhibitory postsynaptic current
Syt1	synaptotagmin 1
Syt2	synaptotagmin 2
Tat	transactivator of transcription
UNC DCM	University of North Carolina Division of Comparative Medicine
UR	unconditioned response
US	unconditioned stimulus
VTA	ventral tegmental area

CHAPTER 1: INTRODUCTION

The world health organization estimates that in 2022 there are currently 38.4 million individuals globally living with human immunodeficiency virus type 1 (HIV). Following the initial discovery of HIV and in subsequent years, the virus was a fatal diagnosis that gradually depleted the body's immune system before progressing to acquired immunodeficiency syndrome (AIDS) and then ultimately death. During the progression to AIDS, the body is not the only part of the patient that is affected. While HIV ravages the body, it also infiltrates and attacks the brain and the fate of many of these early patients was to progress slowly towards a condition known as HIV-associated dementia (HAD) [25, 26]. Research on HAD has revealed the mechanisms by which HIV affects the brain. Briefly, HIV infected monocytes cross the blood brain barrier to enter the central nervous system (CNS) where they release a slew of viral proteins which result in neuronal and astrocytic damages largely thought to underlie the symptoms of HAD [25, 27, 28]. There is no treatment for HAD; however, successful management of peripheral viral replication through combined antiretroviral therapies (cART) slows the progression to HAD [25-28]. While cART is an excellent treatment for the peripheral infection, its failure to penetrate the blood brain barrier makes it a poor medicine for the CNS [26, 27]. The advent of these therapies has demanded a new classification for cognitive disruptions as individuals began exhibiting milder, albeit chronic, neurocognitive symptoms [27].

1.1 HIV-associated Neurocognitive Disorder

Currently, there exist three designations of neurocognitive impairments stemming from HIV infection known collectively as HIV-associated neurocognitive disorder (HAND) [27]. The least problematic of these designation is asymptomatic neurocognitive impairments (ANI) [27]. A person with ANI would largely never notice any disruptions in their everyday life, but when given a cognitive test in a clinical setting, would show reduced cognitive performance compared to their non-HIV infected peers [27]. Slightly more problematic, and of interest in this project, are mild neurocognitive deficits (MND) which involve slightly more noticeable disruptions to the patient's daily life due to the more severe symptoms [27]. Finally, as mentioned above, HAD is the most problematic one compared to ANI or MND [26-28]. Importantly, the neurocognitive deficits stemming from HIV infection are clusters of slowly progressing neurodegenerative conditions whose severity appears to be correlated with the degree of success in managing peripheral HIV replication. For cognitive impairments to advance to HAD, there needs to be sufficient long-term disruptions to immune and blood brain barrier function [29-31]. In other words, HAND is not a distinct condition from ANI, MND, or HAD, but a reflection of the severity of symptoms along a spectrum. As a result of the increasing effectiveness of therapies targeting peripheral HIV infection, the proportion of patients experiencing these less severe symptoms has drastically increased [32, 33]. That is to say, rather than progressing fully to HAD, the greatest proportion of HIV infected individuals with noticeable cognitive impairments fall in the ANI or MND classifications and thus deserve special attention from contemporary research.

HAND is a cluster of cognitive impairments, including executive function, attention, learning, memory, decision making, and verbal fluency, affecting roughly 30-50% of HIV infected individuals [32, 33]. While past research has focused on encephalitis and neuronal loss as being the predominant neuropathological cause of HAND [34-36], contemporary efforts look more at the functional alterations to neurons [35-37]. Changes to synaptic protein dynamics are largely driven by HIV changes to the immunoproteasome that affects the cellular homeostasis and responses to stress [38]. As mentioned previously, HIV infects the CNS through monocyte trafficking across the blood brain barrier. Packaged up in those monocytes is a mixture of HIV-1 viral proteins and toxins that injure neurons and astrocytes when released in the CNS [39, 40]. The independent effects of each of these toxins is being thoroughly investigated and our little slice of this heaven is the transactivator of transcription (Tat). In humans, the severity of symptoms is most strongly correlated with the severity of synaptic disruption and dendritic injury [41, 42]. Of the HIV-1 viral proteins, Tat plays a key role in facilitating structural and functional dendritic defects in neurons [43-46]. HIV-1 Tat's effects on neurons occur through multiple direct and indirect mechanisms [47-55]. Tat depolarizes neurons directly through N-Methyl-D-aspartate (NMDA) receptors [56-58] and can potentiate glutamate induced excitotoxicity [57], resulting in increases in intracellular calcium. Tat can also cause indirect effects on neurons through microglia and astrocytes by stimulating production of proinflammatory cytokines [47] and increasing glutamate release [59]. Of note in this project due to the subject matter, deficits affecting associative learning circuitry: prefrontal cortex (PFC), hippocampus (HPC), and amygdala (AMG), are of special interest.

1.2 Region Specific Effects of Tat Expression

Within the prevue of the PFC, HAND typically results due to deficits in executive function, working memory, attention, and inhibitory control [60-65]. In their 2017 study, Wang and colleagues administered an attention network test to HIV infected individuals and controls [65]. The test is designed to assess three different types of attention including orienting responses, alerting responses, and resolving prediction errors. Orienting responses require the person to detect and then turn towards the origin of a brief stimulus, alerting responses require the person to remain in a vigilant state to detect a series of expected pattern following a warning cue, and resolving prediction errors involves separating signal from noise in a predictable pattern of cues to detect when an "incongruent" or unexpected cue is presented. Their results indicated two important findings. First, participants with HIV showed marked attentional deficiencies in alerting prediction errors. HIV patients were not using the warning cues to speed detection of an expected sequitur as controls did. Likewise, when the pattern following a warning cue was followed by a pattern containing an unexpected stimulus, detection of that stimulus took markedly longer than controls. Secondly, these patients did not show any differences in the speed of orienting responses. Taken together, this means that the detection of stimuli in this cohort was preserved, while the actual processing of that information into anything usable was slowed [65]. Similarly, an animal study from our lab in 2019 showed that mice expressing the Tat protein were unable to use information from their environment to change their behaviors [64]. This study used the Go/No-go task which requires animals to perform a "Go" operant conditioning response, in this case a nosepoke, in order to receive food. However, on some trials an interceding stimulus tells

the animals to not perform that same response in order to receive food. In other words, imagine you are at a stop light and when the light turns green ("Go" signal) another car bursts into the intersection running their red light ("No-go" signal). Despite the green light telling you to go, the safe thing to do is to wait. The efficiency with which the No-go signal can inhibit, or prevent, the behavior is called inhibitory control. In our experiment, the animals with Tat expression showed poorer inhibitory control compared to controls which coincided with an observed increase in spontaneous excitatory postsynaptic current (sEPSC) frequency in the PFC [64]. Notably, a separate study found that spontaneous inhibitory postsynaptic current (sIPSC) frequencies in the PFC were increased to a larger degree in females expressing the Tat protein [66]. The balance of excitatory and inhibitory synapses is critical for the expression of inhibitory control over behavioral processes [67]. Taken together, these studies present two interested findings relevant to the current project. Firstly, in both studies the animals were able to detect the stimuli, thus one can conclude HIV does not impair sensory information for the infected individuals; anecdotally, in the case of our animal study the orienting response to the No-go stimulus was so disruptive it necessitated changing it entirely. Secondly, when it comes to actually using the detected stimuli in a goal-directed task, HIV is detrimental. Tat expression also results in disruptions to calcium signaling vital to PFC long-term potentiation (LTP) and control over behavior [68, 69]. In the case of both humans and animals, HIV disrupts executive function of the PFC which involves the recruitment and coordination of different brain regions to organize behavior [64-66, 68, 69].

For the HPC, HAND manifests as deficits related to spatial memory and associative learning [70-77]. In one 2012 study looking at spatial memory, Cary and

colleagues placed mice with Tat into a Barnes Maze which uses distal spatial cues to guide the animal into picking one of several holes placed on a circular arena. This task requires the animals to not only orient and attend to distal cues, but to also learn that these cues relate to locations in the maze baited with food. Interestingly in their experiment, researchers noted that the presence of Tat resulted in far more errors, and a longer latency to escape (get the food) than controls [70]. While this result clearly showed a spatial learning deficit associated with HIV, one complication with such a procedure is that it involves reward motivation systems since it rewards proper navigation with a primary positive reinforcer, the food pellet. HIV infection also tends to produce anhedonia phenotypes, so any experiment relying on positive reinforcers requires closer inspection [78]. Another study by Fitting and colleagues in 2013 used the Morris water maze task which relies on the negative reinforcement of survival to reward the animal for its successes. In this task the animal is placed into an opaque water and required to use distal spatial cues to determine the location of a slightly submerged platform that offers safety from the water. Thus, the animal is required to use spatial learning to aid its own survival which arguably would be less affected by anhedonia. Nonetheless, researchers found a similar deficit in Tat expressing mice where those mice made more errors and took longer to find the platform than controls [71]. In their study, researchers pointed towards an upset of inhibitory and excitatory synaptic proteins as the cause for disruptions in HPC functioning [71]. Additionally, it seems LTP is inhibited or non-existent in the HPC of Tat expressing mice as well as disrupted pyramidal cell dendritic ultrastructure [71]. These two experiments show a diminished capacity for spatial learning following HIV infection; however, by nature of the tasks themselves, these experiments are complicated

by the use of the PFC to organize a direct sensory information towards goal directed outcomes. Which, as discussed previously, is not the HIV infected brain's best feature. The same Fitting et al. paper performed a contextual fear conditioning task to try to address this complication. In this task the mice were subjected to footshocks in a particular context and then assessed for fear-related freezing behavior the next day when they were placed back in that same context. While this task is again asking subjects to attend to spatial information to inform on expectations, unlike the previous tasks there is no goal. There is no reward for if the animal behaves a certain way, and there is no way for the animal to escape or avoid the footshock. Instead the animal is simply learning what to expect in a certain place and as a consequence the learning shown in this task is not due to any choices on the animal's part, but is instead a reflex. As will be discussed later, the acquisition of this learning is largely PFC independent. The mice with Tat onboard showed less fear than controls at test. Meaning, that when presented with a constellation of cues that predicts danger is imminent, the Tat expressing mice largely ignored these cues. They had failed to connect the dots that the place means danger and failed to alter their behavior accordingly. Much of the damage done by Tat to the HPC comes in the form of altered spine densities in key HPC circuitry [71, 77], as well as alterations to membrane excitability [76]. Taken together, these findings indicate that HAND is marked by HPC related deficits that are independent of any deficits in the PFC.

Finally, the AMG has received far less attention from the HAND community despite some findings indicating it is affected as well. Much like in the HPC, Tat results in reduced dendritic spine density in the basolateral amygdala (BLA), but not the central nucleus of the amygdala (CEA) [79]. Reduction in spine density is thought to underlie most of the

neurocognitive disruptions associated the HAND [27]; and in the AMG results in reduced prepulse inhibition of the acoustic startle response [79]. For associative learning, this finding indicates that predictive and aversive stimuli in the BLA are not being integrated in a way that allows the predictive stimulus to provide information about the imminent aversive stimulus [79]. In turn, this means that CS and US associations in Tat transgenic mice will likely be impaired. Additionally, Tat has been shown to reduce oxytocin, which has anxiolytic effects, in the AMG leading to increased anxiety-like behaviors [80]. In terms of the current study, increased anxiety behaviors may actually lead to an enhancement of the fear response [81]. Nonetheless, as previously mentioned, the Fitting et al. 2013 experiment demonstrated a contextual fear conditioning deficit. It is impossible to talk about fear conditioning and not mention the AMG and as such one question left unanswered by this experiment is whether dysfunction within the AMG may have contributed to the observed fear learning deficit [71]. As will be discussed in a future section, spatial/contextual information is typically handled by the HPC, but the convergence of contextual inputs from the HPC and sensory inputs for the footshock occurs in the AMG which ultimately produces the behavior. Along with the footshocks, discrete cues such as a brief tone or light, are also handled within the AMG. As such, a study that looks at using discrete cues to predict fearful events would largely ignore the impact HIV has on the HPC and PFC to focus on the integrity of intra-AMG circuitry. One study by Hahn and colleagues in 2016 did just that. In their experiment researchers paired a brief tone with a mild footshock on one day, then extinguished the fear response to the tone over the course of the next week. Results from their experiment showed that, unlike contextual fear conditioning, cued fear conditioning proceeds normally among Tat

mice, and in fact it was the control mice that showed a failure to acquire the fear [72]. Meaning that the observed deficits in the Fitting experiments must have been due to the HPC and not the AMG; however, there were several methodological inconsistencies which require further study. Firstly the two experiments used different intensities of footshocks, with the Fitting paper using more intense footshocks. It has long been understood that the intensity of the footshocks is directly correlated with the strength of the fear response and thus may have impacted detection of differences caused by Tat [82]. Secondly, the length and number of exposures to the shocks varied. While the Fitting paper used a single brief footshock, the Hahn paper used four. Again, we understand that the number of presentations directly influences the intensity of conditioned responses and may have obfuscated the findings [82]. Finally, the stimulus presented at test differed in the Hahn paper. During the initial pairings of the stimuli the mice received a 20 s long tone, but at test they received a 200 s long tone. This arrangement is traditionally used to assess latency to cease freezing as a means to judge how strongly generalized the original learning was, but this test was not presented in this manner in their experiment. The results we see for acquisition are more akin to a generalization test than a true test of acquisition. Within that same Hahn experiment was also the extinction learning test. Briefly, during extinction the animal learns that whatever predicted the fear would occur actually isn't the best predictor and thus the fire of fear is extinguished. Extinction is governed by the IL's ability to inhibit projections from the BLA → CEA and thus preventing the production of the fear response. This process typically takes much longer and so in this experiment the researchers used the number of days until the Tat mice and the controls showed a non-statistically significant difference. This

was a curious way to approach this subject since the subjects showed differences between groups on the first day of extinction. The control animals notably showed less than half the fear response they showed at acquisition indicating they did not acquire the fear in the first place. From their experiments the Tat mice took three days to reach a level of freezing similar to the control animals which was labeled as an extinction deficit [72]. This interpretation of the data seems a little dubious, but was nonetheless intriguing given that extinction involves the PFC and appears to corroborate findings from our own research and led to the formation of the current project.

To review, HAND results in a litany of deficits in various regions of the CNS largely through disruptions to dendritic morphology and function due to the Tat protein. With regards to associative learning circuitry, HIV results in HPC dysfunction leading to spatial/contextual learning deficits and PFC dysfunction which results in some potential extinction deficits that do not appear to be the result of a failure to attend to the stimuli. HIV has also been shown to result in AMG loss of dendritic spines and changes neurotransmitter populations [79]. Thus, the current project sought to clean up some methodological differences within the literature to establish a unified methodology for investigating deficits resulting from Tat for the acquisition, extinction, and relapse of conditioned fear responses using a transgenic mouse model of HAND expressing the Tat protein.

CHAPTER 2: TITRATION AND ACQUISITION

In learning, the training histories of stimuli, and their arrangement with other stimuli, are what influence behavior. One of the most basic training procedures is classical conditioning in which a previously neutral stimulus (NS) is paired with an unconditioned stimulus (US) until the response elicited by the US, the unconditioned response (UR), is also elicited by the NS. The NS is now considered a conditioned stimulus (CS), and the response elicited by the CS is the conditioned response (CR) [83]. The CS, when presented, will excite a memory of the US (CS→US) and elicit the CR. For example if you are in an office setting and a coworker brings the group doughnuts every day. Originally you will salivate upon sensing the doughnuts, but you likely will not be salivating once you sense your coworker. Nonetheless you would find that over time the sight of your coworker would cause you to salivate because the coworker and the doughnuts have been paired together so consistently. In this example the coworker is the CS, the doughnuts are the US, and the salivation is the CR and UR respectively and you have acquired a CR to the CS in this acquisition trial. Importantly, the CS does not need to necessarily be a discrete cue such as the sight of a coworker, but can include other stimuli like the time of day, physical place, internal states as CS's. When a discrete cue is used, such as a brief tone or light, the procedure is called cued fear conditioning, while usage of context, such as physical place or internal state, is called contextual fear conditioning. While the brain regions involved in classical conditioning depend on the

sensory modality of stimuli and outcomes, this project will be focusing solely on the circuitry for contextual and cued fear conditioning acquisition, extinction, and relapse.

2.1 Acquisition Circuitry

The involvement of the AMG, PFC, and HPC during Pavlovian fear conditioning is well characterized in the literature; however, the differential involvement of these three regions at different phases of the procedure allows for a focused analysis of the behavioral consequence of HIV-1 Tat-induced damage in each area. At the forefront of any fear conditioning is the role the AMG plays in gating and resolving stimulus inputs [17, 84-87]. The AMG is divided into several distinct substructures which play distinct roles in fear conditioning; principle among these is the BLA and CEA. Despite these two substructures consisting of multiple anatomically distinct substructures, functionally they are considered a package [88]. Our current understanding, summarized in **Figure 1**, paints fear conditioning as a competitive process between tightly interwoven excitatory neurons and inhibitory interneurons within the BLA and CEA [17]. The simplified serial model of AMG function proposes that sensory inputs from the CS and US converge in the BLA where there is a high population of glutamatergic neurons [16]. From there the BLA projects to the CEA; however, these projections are heavily influenced by surrounding clusters of tightly packed GABAergic neurons that are thought to gate information between these two substructures [89]. Within the CEA, the majority of these neurons are GABAergic and work on a system of disinhibition to feed outputs from the CEA to other brain structures controlling the performance of motor functions [17]. The inhibitory interneurons surrounding projections to the CEA from the BLA are subject to neuromodulator influences from the PFC and HPC which become more relevant during

extinction. Their purpose is to code for contextual information and synchronize firing between the HPC and AMG when the contextual information is made relevant for behavioral outputs, i.e. if fear conditioning occurs in multiple contexts or in extinction when learning becomes specific to the context in which the extinction treatment occurs [19]. Additionally, cases in which the context is itself serving as the CS, there are direct projections from the HPC to the BLA which serve the same purpose as the discrete cue CS inputs described previously [20]. Thus, in cases where we are not using the context as the CS, the acquisition of fear memories is an AMG dependent function and as such deficits in the acquisition of cued fear conditioning memories. In cases where we are using the context as the CS, the acquisition of fear memories is dependent on the interaction between the HPC and BLA. Damage, dysfunction, or disruption of these regions results in a reduction of the speed and strength of acquisition of these memories; however environmental factors can also impact these memories [17].

2.2 Current Project

There are many factors which can influence the speed and intensity of acquisition, but most notably for this experiment is the intensity of the US [90]. Current understanding of learning proposes that there is an asymptote to learning, or a cap on how much can be learned about a particular stimulus arrangement [82]. This hypothetical cap or asymptote is met when the animal is no longer surprised that the CS is followed by the US; that the CS has become a good predictor of the US. The intensity of the US contributes to determining both the asymptote of learning and the speed at which an animal reaches asymptote. The more intense a stimulus is, the less trials will be needed to fully learn about the CS→US arrangement, and the more trials will be needed to extinguish this

arrangement later [82]. In the Fitting and Hahn papers previously mentioned, the differing intensities of US may have been one of the contributing factors in discovering a deficit in contextual, but not cued fear conditioning acquisition [71, 72]. Alternatively, it is possible that Tat affects the HPC and AMG differentially and while the AMG functions normally, projections to the BLA from the HPC may be disrupted. To investigate this thread, the methodological differences must be reconciled and the problem approach in a standardized way and this series of experiments accomplished that purpose.

In these experiments we first titrated the intensity of the US and altered the number of presentations to obtain a rate of conditioned responding that both avoided floor effects from not being intense enough, and ceiling from being too intense. Likewise we were looking for a level of conditioned responding that would allow for extinction in future studies as the goal was to keep the methodology as consistent as possible between experiments [91]. Next, we assessed multiple arrangements of contextual and cued fear conditioning to establish Tat induced deficits in acquisition using either of these paradigms. The purpose of these studies was to establish unified methodology and replicate findings from the literature to assess the true nature of the associative learning deficits attributed to the Tat mouse. Given the Tat-induced disruptions to PFC, AMG, and HPC, our hypothesis, based on previous literature, was that, once established, our methodology would confirm previous findings showing deficits in contextual fear conditioning acquisition, and reveal additional deficits in cued fear conditioning acquisition.

2.3 Subjects

Doxycycline (Dox)-inducible, brain-specific HIV-1IIIB Tat1–86 transgenic mice were developed on a C57BL/6J hybrid background as described in detail in previous literature [92, 93]. Tat expression, which is under the control of a tetracycline-responsive promoter controlled by glial fibrillary acidic protein (GFAP) expression, was induced with a specially formulated chow containing 6 mg/g Dox (product TD.09282; Harlan, Indianapolis, IN). Inducible Tat(+) transgenic mice express both GFAP-rtTA and TRE-tat genes, while control Tat(-) transgenic mice express only the GFAP-rtTA genes. At ~4 weeks of age transgenic mice were genotyped to confirm the presence of Tat and/or rtTA transgenes. In all experiments, subjects are grouped by both genotype (Tat(+) or Tat(-)) and by sex (male or female).

Tat expression in experimentally naive adult Tat transgenic mice was induced by Dox treatment starting at 6 weeks of age and continued through the end of the experiment unless otherwise specified. Subjects entered the 10-day experiment at 8 weeks of age resulting in 24 days of Dox treatment in total. All animals were bred by the University of North Carolina Division of Comparative Medicine (UNC DCM) and were group housed under a 12/12 h light-dark cycle. The colony room temperature was maintained at 21°C and 32% humidity. All animal procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC) and followed The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines.

In the shock titration experiment, Tat expression was not induced. These experiments used 16 Tat(-) subjects (males and females, counterbalanced) grouped by the intensity of shock they received. Only Tat(-) subjects were included to examine how control subjects would respond to varying US intensities [90]. All subjects were run simultaneously one hour prior to the end of their dark cycle. The contextual fear conditioning experiment with the four .4 mA shocks used 8 subjects per group while the contextual fear conditioning experiment with the single .7 mA shock used 8-10 subjects per group. Finally, the cued fear conditioning experiment used 7-9 subjects per group.

2.4 Apparatus

Standard mouse experimental chambers (MED Associates ENV-307W) were housed in sound and light attenuating cubicles (MED Associates ENV-022MD). A 28 V DC, 100 mA house light (MED Associates ENV-215W) was mounted on the wall of the chamber and was illuminated during all phases of these experiments. The US was delivered using standalone aversive scramblers (MED Associates ENV-414S) connected to gird floors (MED Associates ENV-307W-QD) via shock output cables (MED Associates SG-219G-10). In all experiments, US duration was 2 s, and unless otherwise specified occurred at .7 mA intensity. In the cued experiment, CS presentations were delivered using a Sonalert module with volume control to deliver an 80 dB 2900 Hz tone (MED Associates ENV-323AW). Sessions were recorded using Amcrest FullHD 1080P 2MP Dome cameras mounted to the ceiling of the light and sound attenuating chambers. These cameras recorded to an Amcrest Security Recorder (AMDV8M16-H5). Behavioral testing occurred in a dark room illuminated by red fluorescent lighting and all testing

occurred with white noise from an air conditioning unit located inside the room. The testing room was kept at 22°C room temperature with 30% humidity.

2.5 Procedure

For the shock titration experiment, following Tat induction and during acquisition, Tat(-) mice were placed into chambers for a 3 min habituation period before the first unsignaled stimulus presentation. In the first group, subjects received four .4 mA shocks with a variable inter-stimulus interval (ISI) of 3 min (2-4 min). The second group received two .4 mA shocks on each day for two days with an ISI of 6 min. This group was started one day prior to all other groups to align testing times. The third group received two .6 mA shocks with a 6 min ISI. Finally, the fourth group received two .8 mA shocks with a 6 min ISI. All subjects were removed from the chambers 2 min following the last stimulus presentation for a total session time of 15 min. Following acquisition testing was conducted by placing subjects back into the same experimental chambers used in acquisition for 15 min. Freezing behavior from this session was used to populate the present data as described below.

In the contextual fear conditioning experiment using the .4 mA unsignaled shocks, all subjects were placed into the chambers for a 3 min habituation period prior to the first stimulus presentation. Subjects then received four .4 mA shocks with a variable ISI of 3 min (2-4 min). As before, subjects were removed from the chambers 2 min after the final shock delivery for a total session time of 15 min. The next day, subjects returned to the same chambers for 15 min observation with no US delivery. Freezing behavior from this session was used to populate the present data as described below.

In the contextual fear conditioning experiment using the .7 mA unsignaled shock, training proceeded as previously described in the .4 mA contextual fear conditioning study. However, only a single presentation of the US occurred after a 3 min habituation period. As before, subjects were removed from the chambers 2 min after the final shock delivery for a total session time of 5 min. The next day, subjects returned to the same chambers for 5 min observation with no US delivery. Freezing behavior from this session was used to populate the present data as described below.

Finally in the cued fear conditioning experiment, subjects proceeded as previously described in the .7 mA contextual fear conditioning study. However, following the 3 min habituation phase, a 60 s tone CS preceded the US. Subjects were removed from the chambers 1 min after the final shock delivery for a total session time of 5 min. The next day, subjects returned to the same chambers for 5 min observation with no US delivery. Freezing behavior from this session was used to populate the present data as described below.

2.6 Statistical Analysis

Power analyses based on previous studies with the Tat transgenic mouse model determined animal counts. All statistical analyses were conducted using two-way analysis of variances (ANOVA) with Sex (2 levels: male, female) and Genotype (2 levels: Tat(-), Tat(+)) as factors, followed by Bonferroni post hoc tests if necessary. An alpha level of $p < .05$ was considered significant for all statistical tests used. Unpaired and paired Student's *t*-tests were conducted when appropriate and necessary to examine individual group differences from baseline freezing values gathered on Day 1 of each experiment.

These studies did not examine estrous cycle as part of the inclusion of female subjects. The stress caused by daily vaginal lavage had great potential to affect conditioned emotional responses elicited from behavioral training. Experimental manipulations were delivered after counterbalancing subjects for age and sibling status. Animals were distributed into groups randomly. Experimenters were blind during both data gathering, and analysis at all stages of the experiment.

Freezing behavior was quantified using the bin method for hand coding. Session time was divided into 5 s bins and coders indicated whether or not the subject exhibited freezing behavior, defined as no motion for >1 s, in a binary manner. Inter observer agreement for >85% of bins on 30% of all videos for each phase was used to ensure reliability of coding. In contextual experiments, percentage of bins in which the animal freezes are the dependent measure. In cued fear conditioning experiments, the dependent measure will be the percentage of bins in which the animal freezes during the CS only. All descriptive statistics are reported as means (M) ± standard error of the mean (SEM).

2.7 Results

We first sought to titrate the unconditioned stimulus in our studies with the goal of producing a conditioned response that was both robust enough to avoid floor effects while moderate enough to allow for extinction in our Tat transgenic mouse line of HAND. These mice are able to express the Tat protein when fed a doxycycline infused chow. Tat(+) mice express the Tat protein while Tat(-) mice do not. We placed control (Tat(-); *n* = 8/group) animals into Standards Med Associates operant chambers equipped to deliver alterable intensities of 2 s footshock US. Animals entered the chambers on the first day

and were exposed to either four .4 mA shocks on one day, two .4 mA shocks on two days, two .6 mA shocks on one day, or two .8ma shocks on one day. A higher milliamperage and more shock presentations are known factors that modify the strength of conditioned responses. Intensities and number of presentations were chosen to reflect previous literature [71, 72]. This experiment demonstrated success in producing a conditioned response measured 24 h following acquisition (**Figure 2A**). The group that received four .4 mA shocks on one day showed decent acquisition (*M* = .48, *SEM* = .11) while simultaneously resulting in the least stress of any of the four conditions and thus was selected for US delivery in contextual fear conditioning experiments.

Following titration experiments, we attempted to replicate previously established contextual fear conditioning acquisition deficits associated with the Tat transgenic mouse model [71]. In our first procedure, we used the US delivery established in the titration experiment. On Day 1, Tat(+) and Tat(-) male and female (*n* = 8/group) subjects were placed into the chambers and presented with four .4 mA shocks. On Day 2, subjects were returned to the chambers and freezing behavior was assessed. There were no main effects of genotype or sex on freezing behavior ($p > .05$; **Figure 2B**). A second experiment investigated if a single more intense US presentation would replicate previous deficits. Now, on Day 1, subjects (*n* = ~9/group; 8-10) received a single .7 mA shock. On Day 2, subjects were returned to the chambers and freezing behavior was assessed. There were no significant main effects of genotype or sex on freezing behavior ($p > .05$; **Figure 2D**).

Upon failure to replicate contextual fear conditioning deficits, we assessed cued fear conditioning using the single .7 mA shock. On Day 1 subjects (*n* = ~8/group; 7-9) were placed into chambers. After a 3 min habituation period, a single 60 s 2000 Hz tone

CS was presented. At the termination of the CS, the US was presented. Subjects were allowed an additional min in the chambers following US termination. On Day 2, subjects were returned to the same context and presented the same CS at 3 min. Freezing behavior during the CS presentation was assessed. There was a main effect of sex on freezing behavior during the CS, F (1, 30) = 8.326, p = .007 (**Figure 2F**). Bonferroni post hoc tests revealed that female Tat(+) animals exhibited significantly less freezing; female Tat(+) mice froze less (M = .389, SEM = .161) than male Tat(+) mice (M = .798, SEM = .048), p = .029, and male Tat(-) mice (M = .767, SEM = .083), p = .021. Overall these acquisition studies indicate Tat transgenic mice show a deficit in one-trial cued acquisition and extinction learning, specifically Tat(+) females. Further experiments will explore ABC renewal and reinstatement to investigate potential deficit in these forms of relapse.

2.8 Discussion

This collection of studies represents the initial preliminary experiments to establish the methodology in future experiments. Principally, these studies were to gauge the type of US to provide to the Tat transgenic mouse that would allow the subjects to produce a measurable freezing response that was neither too extreme to extinguish nor too weak so as to not produce any fear response. Concurrently, the US was titrated to result in the least amount of aversion to the subject while still establishing a CR. Furthermore, these studies were designed to assess deficits in acquisition of the CR due to Tat expression. In our experiments we established a cued fear conditioning deficit in female Tat(+) mice, but did not see any such deficit for contextual fear conditioning.

Shock titration experiments are chiefly used when first using a fear conditioning procedure in a laboratory setting [94]. Whenever using a new animal model, equipment, or a new setting it is important to ensure the US is being delivered consistently across all subjects, and that all subjects are experiencing the shock consistently. Subjects in the shock titration experiment (**Figure 2A**) demonstrate a typical sensitivity to US intensity, frequency, and spacing that one would expect from wild type animals [82]. As the number of presentations or the mA of the shock increase, so does the CR. In our experiment we determined that subjects achieved sufficient levels of freezing to avoid the aforementioned ceiling and floor effects, while not overtraining, using four .4 mA shocks. Following data from the contextual fear conditioning studies, this value was changed to one .7 mA shock to achieve a similar effect while more closely replicating previous literature [71]. As this study only employed Tat(-) control subjects, this experiment provided a solid foundation for the suitability of Tat transgenic mice in fear conditioning and allowed cataloguing and accounting for equipment variability.

Using data from the shock titration experiments, a new cohort of subjects underwent a four-trial delay contextual fear conditioning paradigm. This experiment attempted to replicate the original contextual fear conditioning study established in previous literature with the Tat transgenic mouse model [71]. Given that the US intensity in the current study was much lower than reported in literature, the next step was to increase the US intensity. Simultaneously, the number of shocks was reduced to one .7 mA shock to avoid ceiling effects in which over-trained acquisition memories retard subsequent extinction [95]. Subsequently, subjects showed similar low-level freezing behavior at test. Keep in mind that during these studies, the context serves as the CS

and the data shown reveals the CR to that CS. Thus these two findings, along with renewal data discussed later, indicate that our Tat transgenic mice, including Tat(-) controls, seem to have a lack of sensitivity to the contextual information. While the HPC deficits in Tat transgenic mice have been well documented [70-75], the finding that Tat(-) subjects also performed poorly was surprising. One explanation for the poor performance displayed by our Tat transgenic mouse line is the finding of a previous study that showed strain differences in sensitivities to contextual cues during the fear conditioning procedure [96]. In these studies, DBA/2 mice demonstrated normal acquisition to cued fear conditioning but showed a stark deficit in the performance of CR's to contextual CS's. This deficit persisted independent of environmental and methodological alterations to the fear conditioning procedure [97]. Similarly, previous literature has shown strain dependent differences in HPC long-term potentiation and spatial memory in several inbred and transgenic strains compared to C57BL/6J mice [98-101]. Therefore, further studies are needed to investigate whether Tat(-) subjects show a comparable CR profile to the C57BL/6J mouse strain from which they are derived. It is entirely possible, that much like the DBA/2 mice, the Tat transgenic mice are simply not a proper strain to use as controls in contextual fear conditioning experiments. As such, future studies may want to use wild-type C57BL/6J mice as controls, to account for influences from the Tet ON system used to express Tat from astrocytes.

The final experiment in this series examined Tat transgenic mouse responses to single trial cued fear conditioning. This experiment established an apparent deficit in female Tat(+) animals in which they showed a much weaker CR at test than their male counterparts or control animals. In a departure from previous experiments, this one

preceded the US with a 60 s auditory CS, and as such the data represents solely the CR during the CS. Traditionally, studies that have shown Tat's effects on the AMG show deficits in male subjects [79, 102, 103]; however several factors may have resulted in the present data. Firstly, these experiments were run towards the end of the subject's night cycle. Studies looking at the effects of circadian rhythm on stress show that in males, corticosterone levels are significantly higher at the end of the dark cycle than at the beginning [104]. In this way, our male subjects were perhaps showing greater baseline levels of stress than females. Within fear conditioning literature, stress enhances intensity of CR to fearful stimuli and as such may explain why males presented a stronger fear response [105-107]. Studies examining the relationship between sex, stress, and fear conditioning have found sex to differentially impact the relationship between stress and fear [106, 107]. In males, stress clearly enhances fear conditioning learning, while in females the results are mixed [106, 107]. In acquisition, females tend to show moderate enhancements, or none at all, largely depending on the type of stressors used [106, 107]. In general, females tend to show enhanced fear responses only when under social stress, which was not a stressor used in the current series of experiments [108]. Additionally, previous literature shows Tat dysregulates the hypothalamic-pituitary-adrenal (HPA) axis resulting in increased corticosterone in males [109]. Indeed the circadian rhythm dependent increase in corticosterone may have had an additive effect with the already heightened levels in Tat(+) to obscure any deficits related to learning in this design. As will be discussed later, subsequent studies that occurred towards the beginning of the night cycle failed to replicate this observed deficit in female Tat(+). This failure to find differences in those studies was notably not due to an increased CR in Tat(+) females,

but to a reduction of the CR exhibited by all other groups. A second influential factor may have been environmental stressors pre- and postnatally. These experiments occurred near the beginning of the COVID-19 pandemic which saw several changes to the home cage environments of these animals. Notably, a dynamic animal care staffing and the relocation of colony breeders preceded the cued fear conditioning experiment. In other words, this cohort of animals was exposed to a unique cocktail of prenatal stressors that are known to affect behavioral stress responses later in life [110]. Specifically, exposure to unknown male mouse odors during weaning led to higher behavioral measures of stress later in life for male mice, but not female mice [110]. In the current experiment it is highly probable that relocation of colony breeders to a novel environment introduced such a stressor which also contributed to the high levels of CR we saw in our male animals.

Nonetheless, this experiment observed a transient acquisition deficit in female Tat(+) subjects. Within acquisition, circuitry involving the PFC and AMG are critical for the production of fear responses following acquisition [17, 84-87]. The balance of excitatory and inhibitory activity within the PFC is the primary contributor for AMG disinhibition and as such changes to the balance of these synapses caused by Tat may help explain acquisition deficits [17, 19]. In female Tat(+) mice, the frequency of sEPSCs are moderately increased [64]; whereas, female Tat(+) subjects show increased sIPSC frequency compared to Tat(-) females [66]. Thus, this imbalance would mean the inhibitory tone of that system was preventing disinhibition of BLA to CEA intra-AMG circuitry, resulting in the observed acquisition deficits in female Tat(+) subjects [17]. In previous literature, the imbalance seen in males is quite different. While sEPSCs frequencies are similarly increased, sIPSC frequencies are decreased [66]. As such, for

our male subjects they likely had an imbalance largely favoring excitatory synapses. At test, this sex dependent difference in PFC activity led to males having enhanced fear response while females showed reduced CR compared to all other groups.

In summary, the findings of the acquisition studies revealed a cued, but not contextual, fear conditioning acquisition deficit in female Tat(+) transgenic mice. It appears from this series of experiments that the Tat transgenic mouse line as a whole is not sensitive to contextual information and as such did not attend well to the contextual information in these experiments. To support this finding, future experiments should use additional control groups as discussed later. The observed deficits in cued fear conditioning for Tat(+) females seem to stem from the imbalance of excitatory and inhibitory synapses in the PFC [17, 19]. These studies succeeded in their role to establish consistent methodology to be used in subsequent experiments and establish a transient acquisition deficit in female Tat(+) subjects.

CHAPTER 3: EXTINCTION AND RENEWAL

With an observed acquisition deficit in our Tat(+) female mice for one-trial cued fear conditioning, the next step was to investigate the supposed extinction deficit first reported by Hahn et al. 2016 [72]. Extinction involves explicit un-pairings of the CS and the US, and in the case of cued fear conditioning, this is accomplished by presenting the CS without the accompanying US. Central to the understanding of extinction, is the notion that this process does not erase acquisition memories, but rather retroactively interferes with the performance of the CR [21]. In other words, extinction is not a form of unlearning but of new learning, and as a result the originally conditioned memories remain despite extinction and are vulnerable to relapse [21].

3.1 Extinction Circuitry

The circuitry governing extinction learning, as summarized in **Figure 3,** is complex and involves a distributed network of neuromodulator interactions that gate intra-AMG signaling [111]. Early studies probed the AMG's role in extinction using lesions and pharmacological inactivation; however, the lesion studies were problematic given that a lesion of the AMG would disrupt the performance of the CR regardless of if extinction was involved or not [112]. As techniques improved, more focused lesions revealed that even when the acquisition circuitry remained intact, lesions to the AMG were unable to disrupt extinction [113]. Pharmacological activation of GABAergic activity in the AMG before extinction training likewise was unable to prevent extinction within a session [114];

however, GABA agonists administered following an extinction session were able to facilitate extinction learning [115]. This data is supplemented by findings that following extinction, GABA receptor associated synaptic protein Gephyrin is upregulated indicating there are structural changes to GABA receptor expression in the AMG following extinction learning [15]. These changes have been attributed to NMDA dependent plasticity within the AMG as NMDAR antagonists were able to disrupt impair extinction retrieval but not within session extinction [116]. So the question becomes, what is driving the NMDAR activity?

The infralimbic cortex (IL) of the PFC was first implicated as the driving force following lesions studies which showed that ablation of the IL prevented extinction retrieval the next day, but did not impair extinction learning [15]. A multitude of studies pharmacological inactivation of IL function (Na+ channel blockers, NMDAR antagonists, protein kinase A inhibitors) have found similar functions in that the within session extinction is not affected, but the retrieval of extinction learning the following day was impaired [12-14]. Further studies show that there are physiological changes to the function of IL neurons following extinction which underlies performance of extinction memories within the AMG. Research has found that the degree high-frequency bursting of IL neurons after extinction training is correlated to the degree of extinction retrieval observed the following day; more bursts means more extinction [13]. Secondly, the inhibitory tone of the IL was correlated with the level extinction retrieval observed the following day [18].

Interestingly, extinction learning is highly specific to the context in which the treatment occurs [5]. The HPC encodes contextual information which project to the PFC

and the AMG [22]. Disruptions to this circuitry through lesions or pharmacological inactivation can disrupt, but not eliminate, the retrieval of extinction memories [24]; the subjects were able to extinguish within a session, but showed poor extinction retrieval the following day. Importantly, inactivation or lesions to the HPC are not sufficient to prevent extinction retrieval, rather the HPC seems to gate the performance of extinction based on how closely the retrieval context matches the training context.

Taken together the literature shows a clear circuitry for extinction. Within the AMG, CS and US convergence results in the production of a fear response. At the same time, contextual inputs from the HPC innervate the PFC and AMG providing contextual information necessary to resolve behavioral outputs from the AMG. The IL then takes converging information about the CS from the AMG and the context from the HPC. IL activation of populations of GABAergic interneurons within the AMG to inhibit the output of the CEA to prevent the performance of a fear response is warranted given current stimulus conditions.

3.2 Renewal Circuitry

As previously mentioned, extinction training is highly context specific. So much so that any departure from the extinction context will be met with a relapse in the CR known as renewal [21]. The circuitry for renewal is summarized in **Figure 4**. First the animal learns an excitatory association (CS→US) in acquisition before it learns a second association (CS→No US) during extinction. There are several paradigms used to study renewal. "ABA renewal" is when conditioning occurs in context A, extinction in context B, and then the animal is returned to the conditioning context, A, for testing [1, 2, 5]. In "ABC

renewal" conditioning and extinction occur in A and B, respectively. The animal is then tested in a novel context, context C, and the effect is smaller but still occurs in this paradigm. This paradigm allowed researchers to determine that it was the departure from the extinction context, and not the return to the conditioning context, that resulted in relapse [5]. The role of the context and subsequently the HPC is of special importance in renewal.

As one author rather humorously stated, there is "general consensus" that the HPC is involved in the renewal of conditioned fear. Which is a rather humble way of stating: the HPC is necessary to observe renewal [3, 4, 6]. Muscimol injections to the dorsal HPC are sufficient to prevent the renewal effect [4]. In their study, Corcoran and Maren were able to demonstrate a reversible ablation of the renewal effect using Muscimol injections. Rats acquired and then extinguished a conditioned response. At the renewal test, acute muscimol injections were able to block the renewal effect; however, tests in the same animal the next day without muscimol on board showed normal renewal. In this same experiment, researchers showed that muscimol injections to the HPC do not prevent freezing responses in unextinguished subjects. Muscimol injections to the HPC prevent the contextual gating of extinction learning while preserving the freezing responses. Interestingly, the HPC is not the only region which, when disrupted, can impair the renewal effect. The prelimbic cortex (PL) has been implicated in a similar manner in which pharmacological inactivation of the PL after extinction prevents renewal response [6]. Even more interestingly, in this study disruptions of the PL prior to conditioning did not impact acquisition or extinction and only affected the contextual gating of extinction learning. As the PL is innervated by the HPC, and itself innervates the CEA, this suggests

an important role for the PL in discriminating between contexts after extinction has occurred and reinforces the idea of direct connections between the HPC and PL being necessary for renewal. However, the PL is not the sole target of the HPC, there are also projections from the HPC to the BLA which have been implicated in renewal [23]. In this experiment, researchers correlated c-Fos expression in the BLA, HPC, IL and PL following renewal tests. Findings indicated that there was significantly more c-Fos activity in BLA projecting neurons from the PL and HPC than in the IL. This shows that while the IL is important for extinction retrieval, it seems the PL is much more important for renewal. These results also indicated significantly more c-Fos activity along the HPC→PL→BLA pathway than the HPC→BLA pathway but disconnection of either pathway prevented renewal. Finally, while there was significantly more PL activity than IL, there was still significantly more activity than baseline in the IL during renewal tests which supports the notion of the performance of conditioned responses resulting from an ever-present competition between excitatory and inhibitory processes within this circuit.

To summarize, there currently exist two pathways by which the HPC gates contextual control of extinction memories. First, there exists a direct pathway between the HPC and BLA which is necessary for the renewal effect; however, this pathway sees relatively low activity compared to a second necessary pathway between the HPC, PL, and BLA. Likewise, there is HPC→IL activity that is recruited during renewal that seems to be out competed by the strong PL activity driven by a mismatch between extinction and testing contexts [3-6]. Consequently, examinations of renewal deficits in our Tat transgenic mice present an opportunity to examine a pathway that is unique to both acquisition and extinction, as well another form of relapse discussed later: reinstatement.

3.3 Current Project

Previous literature reported an extinction deficit in Tat(+) transgenic mice; however, the methodology and data from that study did not warrant this conclusion [72]. Nonetheless, given previous data on how the HPC and PFC are affected in Tat transgenic mice and the importance of these structures in extinction, it is very probable there is an extinction deficit [60-65, 70-75]. As previously mentioned, in the PFC the balance of excitatory and inhibitory synapses is disrupted [17, 19, 64, 66]. Inhibitory activity in the IL is critical for the suppression of the fear response in the AMG during extinction [12-15, 18]. Thus, the current project sought to examine this possibility with a proper examination of extinction. Central to this examination is the understanding that there are acquisition deficits which need to be accounted for. First, a direct comparison between groups for raw conditioned response intensities, such as that in previous literature (i.e. [72]), ignoring the fact that the intensities on Day 1 of extinction are already very different between groups. In fact, this would likely provide the mistaken conclusion that Tat is an extinction enhancer since the conditioned response intensity is already so low. Likewise, a comparison of the rates of extinction are equally as improper. While ultimately a flawed model, the Rescorla-Wagner model can provide insights into why this is so [82]. The product in the model is the change in learning that occurs trial to trial. Among many factors surrounding learning, the model accounts for the current "level" of learning as being particularly influential on the change in learning between trials. To summarize briefly, if the subject already has not learned much about the CS→US relationship, then the change in learning on each trial will be much less than a subject that has reached asymptote for learning during acquisition. In terms of

the current project, this would lead one to conclude mice showing an acquisition deficit were also showing an extinction deficit; however, if the extinction deficit is simply a product of the acquisition deficit then the conclusion would be misleading. The method we employed here examined whether there are differences in conditioned responding intensity on the final day of extinction relative to the level on the first day. In other words, we are comparing the proportions of conditioned responding remaining after extinction between groups. As it does not affect the extinction process, these experiments also examined renewal following extinction.

While findings from our previous experiments did not indicate an acquisition deficit in contextual fear conditioning, the large body of literature supporting HPC dysfunction in Tat transgenic mice, including contradictory results in another contextual fear conditioning study [71], warranted further review at a more subtle way in which the animals may be using context: renewal [70-75]. Again, in renewal subjects experience a relapse in their conditioned responding due solely to a departure from the extinction context. So in these experiments, acquisition occurs in one place, Context A, extinction in another, Context B, and finally the renewal test occurs in either the acquisition context or a novel context, Context C. When subjects are returned to the acquisition context, this is known as ABA Renewal, and when placed in a novel context this is known as ABC Renewal. In contextual fear conditioning, it is thought that direct connections between the HPC and BLA are what account for learning the conditioned response [20]. In contrast, while renewal circuitry requires this connection, the c-Fos data shows an overwhelming amount of activity is routed through the HPC→PL→BLA pathway. Likewise these connections are required to demonstrate renewal. Thus, while the same pathway, as in contextual

fear acquisition, is engaged, there is also a different PFC related pathway engaged which could be differentially affected by Tat. Given the upset balance of inhibitory and excitatory synaptic proteins functioning and diminished LTP in the HPC in Tat transgenic mice [71], it is very likely we will see impaired contextual effects commonly seen during associative learning [71]. Consequently, the current project examined extinction and two types of renewal to assess both PFC and HPC related deficits related to the presence of Tat in our Tat transgenic mouse line. We hypothesized, based on the literature that Tat's effects on neurons in the HPC and PFC would result in an observable behavioral deficit in extinction and both types of renewal.

3.4 Subjects

Subjects were used as previously described in section 2.3. The ABA renewal experiment used ~6 subjects (5-8) per group while the ABC renewal experiment used 8 subjects per group. All subjects were run 1-2 h after the beginning of their dark cycle.

3.5 Apparatus

These experiments used the same apparatus as previously described in section 2.4. Context A was a darkened chamber with the house light off, while Context B was a separate illuminated chamber with the house light on. Context C was a separate darkened chamber with a peanut odor cue placed inside the sound and light attenuating enclosures.

3.6 Procedure

Acquisition in Context A and session/stimulus timing proceeded as previously described in section 2.5. Following acquisition, extinction training involved 7 days of non-reinforced presentations of the 60 s CS once per session in Context B. As before, subjects were allowed 3 min habituation time before the CS was presented. At test, in the ABA experiment subjects were returned to context A and once again presented with the CS at 3 min; while, in the ABC experiment subjects were moved to novel Context C.

3.7 Statistical Analysis

Acquisition data was analyzed as previously described in section 2.6. For extinction, a proportion of CR remaining value was calculated using the following formula: (final day of extinction CR) / (first day of extinction CR). In this way, the measurement was sensitive to any changes in acquisition deficits. Likewise, for relapse measurements, the following formula was used: (test day CR) / (last day of extinction CR) to find the proportional increase in the CR due to renewal. As before, this measurement would be sensitive to any deficits seen in extinction. All statistical analyses were conducted using two-way analysis of variances (ANOVA) with Sex (2 levels: male, female) and Genotype (2 levels: Tat(-), Tat(+)) as factors, followed by Bonferroni post hoc tests if necessary. An alpha level of $p < .05$ was considered significant for all statistical tests used.

3.8 ABA Renewal Results

Undeterred by the lack of contextual fear conditioning deficits, we still wanted to use a behavioral task that would tax the HPC to probe for differences in contextual processing that may be more subtle than a shock to the paws. Thus, we sought to employ

the renewal test in which animals use contextual information to disambiguate information about the CS following prediction errors. In other words, in this task subjects are required to not only detect and respond to the cue, but recognize the place in which that cue is being presented and behave accordingly. As previously mentioned, any departure from the extinction context should be sufficient to produce a renewal effect. In this experiment, context A was a dark chamber while context B was a separate illuminated chamber. Acquisition occurred in context A, same as previously described. On Day 2, subjects (n = ~6/group; 5-8) were transferred to context B and underwent 7 days of extinction before returning to context A on Day 9 for a renewal test. Freezing behavior on Day 2 is indicative of acquisition learning, while the change in freezing behavior between Days 2 and 8 is indicative of extinction learning. Finally, the change in conditioned responding between Days 8 and 9 is indicative of renewal. Given that there were differences in acquisition learning, and the possibility for differences in extinction learning, it was necessary to assess extinction and renewal relative to each subject's past performance. There was a main effect of genotype on acquisition learning, F (1,24) = 4.927, p = .038, and extinction learning, F (1,20) = 4.560, p = .045. There were no significant main effects for the renewal test, p > .05. Bonferroni post hoc analysis did not reveal any significant differences (p > .05; **Figure 5B)**.

Interestingly, this experiment appears to show a reversal of the previous finding demonstrating the acquisition deficit in female Tat(+) subjects to now show an acquisition deficit in male Tat(+) subjects; however, a comparison of the two experiments yields two conclusions. First, the level of conditioned fear demonstrated by female Tat(+) animals remains more or less consistent between the two studies while the male Tat(+) subjects

seem to vastly change between the two studies. As will be discussed in a future section, this could be due to methodological or environmental variability between the two experiments. Namely, the acquisition studies were done in the evening while this renewal experiment was performed in the morning. Additionally, previous literature from our own lab has demonstrated that chronic stress does not uniformly impact Tat(+) males and females [64]. During this period, there were changes to animal care staff, breeding procedures, and transportation environment, which all may have contributed to a stressful situation outside of the experiment for our subjects in this particular experiment.

Another interesting finding from this data is the lack of renewal effect in our Tat(-) subjects. We originally hypothesized based on previous literature, that the Tat(+) mice would have disruptions to hippocampal functioning which would lead to a failure to process contextual information and thus would not be sensitive to the changes in context necessary to observe renewal. Thus, we expected Day 9 to show a level of freezing similar to that of Day 2 (**Figure 5B**) for our Tat(-) subjects and to reveal a lack of relapse in our Tat(+) subjects. Instead, none of our animals seemed to show a renewal effect which left us with two options. First, perhaps the transgenic line itself is incapable of renewal; previous research has shown certain in-bred mouse lines demonstrate acquisition and extinction, but fail to show renewal due to a lake of sensitivity to contextual information[117]. Second, as will be discussed further in a future section, maybe the contexts themselves were not salient enough for the subjects to attend to; the data shown in **Figure 5** is more similar to what an extinction graph would look like if you never changed the context within the experiment. In other words, if we make the renewal

context especially salient and distinct then maybe we could observe the renewal effect in our mouse line. This led us to a second type of renewal, ABC Renewal.

3.9 ABC Renewal Results

For ABC renewal, rather than returning the animal to the acquisition context, you place the animal in a novel context following extinction. Again, since renewal occurs due to a departure from the extinction context, a move to a new place should be sufficient to produce the renewal effect. As previously described, this experiment (n = 8/group) proceeded the same as the ABA renewal experiment, except for the novel context on Day 9. This new context C involved the animal being placed in a new chamber with a novel peanut oil odor permeating the chambers. The calculus was to use the mouse's primary sense to present an extremely salient olfactory stimulus to make it nigh impossible for the mouse to not realize they are in a different context. For this experiment, there were no significant effects for sex or genotype on acquisition, extinction, or renewal (all p's >.05; **Figure 6B**). There was a trending main effect for sex on extinction learning, p = .081, largely driven by a single Tat(-) female mouse somehow performing a conditioned response 250% more intense on Day 8 than it did on Day 2. Taken together this study indicates that all subjects acquired and extinguished their conditioned responses, as well as achieving similar performance on the renewal test.

Notably, much like with the ABA Renewal experiment, the ABC Renewal experiment failed to produce a renewal effect. While possible, it would be hard to argue that the subjects were unable to perceive the change in contexts. One option could be that the subjects were not exposed to the contexts for long enough to fully appreciate the

constellation of stimuli before them. While our animals did not show sensitivities to contexts, conditional place preference studies have shown context sensitivities in Tat transgenic mice using longer periods of exposure to the context [118]. However, it seems more likely that the Tat transgenic mouse line may simply be the wrong strain of animal to observe renewal. The genetic differences between the Tat transgenic mouse and its C57BL/6J progenitors would be a good target for future analysis of the neural machinery necessary to observe renewal.

3.10 Discussion

These experiments examined the effects of Tat on the processes of extinction and renewal. The ABA renewal experiment revealed acquisition and extinction deficits in male Tat(+) animals which supplements previously established findings showing contextual deficits in male Tat(+) mice [71]. This group performed a less intense CR during the acquisition test and did not see a change in the CR following extinction. In males, Tat expression in the AMG results in decreased dendritic spine density in the BLA but not the CEA [79]. Taken together with previously mentioned depression of sIPSC activity in the PFC in male Tat(+) mice [66], one possible explanation for the observed deficit is disrupted BLA activity resulting in failure to encode acquisition memories. As decreased frequency of sIPSC is only relevant for the expression of fear memories and should in fact enhance fear responses owing to the lower inhibitory tone within the PFC [17], encoding failures must explain the observed deficit. Interestingly, the Tat(+) females did not show an acquisition deficit as they had in the cued acquisition study described in Chapter 2; however, the amount of freezing exhibited by this group stayed consistent between the two studies. Instead, it was all of the other groups that showed reduced

levels of the CR that accounted for this apparent erasure of cued acquisition deficits in Tat(+) females. As mentioned previously, these studies were performed at the beginning of the animals night cycle and as such their corticosterone levels in males were likely much lower than in the previous study [104]. Additionally, the environmental stressors that existed in the previous study showing female deficits were no longer present [110]. Taken together, these subjects were likely experiencing less background stress that may have contributed to the less intense CR from all groups. An additional difference was that the acquisition test took place in a different context than the fear was acquired in. Even during cued fear conditioning experiments, the animal is always receiving contextual information that will receive some of the associative value of the US [82]. Although sex differences in how rodents generalize contextual CRs following acquisition typically conclude males generalize fear across contexts more than females [119-121], this pattern was not consistent with the current data.

The data from the ABA renewal study also reveal a stark extinction deficit in the same group of male Tat(+) mice. This measurement shows us that, relative to the CR at the beginning of extinction, the CR for male Tat(+) mice on the last day of extinction represents less change in the CR than in other groups. In males, this finding may be the result of the aforementioned decreases in sIPSC frequencies in the PFC [66]. Inhibitory activity within the PFC, and specifically the IL is directly responsible for the suppression of fear responses within the AMG [12-15, 116]. Thus, less inhibitory tone in the PFC is likely the root of the failure of male Tat(+) subjects to extinguish; however interestingly previous literature points towards females typically showing greater resistance to extinction treatments [122-125]. However, findings from Gruene et al. showed that

females actually demonstrate enhanced extinction due to sex differences in fear responses [124]. In their study they found that analysis of conditioned freezing obscured measurements of female CRs to the CS. Many, but not all, female rodents exhibit darting behavior while many, but not all, male rodents typically show freezing behavior. While clearly we do observe our females freezing, the authors in that study make the point that while the freezing measurement extinguishes rather quickly, the darting response could remain for much longer [124]. Applied to this study, if we had been equipped to measure the speed and frequencies of darts following CS presentations, we may have been able to see an extinction deficit in our Tat(+) females as well. This failure of the current project highlights a need for future studies using the Tat transgenic mouse to account for sex differences in CR phenotypes. Furthermore, this phenotypic difference may be an important grouping variable for determining specific subpopulations, darters and freezers, that might be differentially affected by Tat given the wide variability in freezing observed in these experiments.

Renewal is a return of the CR brought about by a departure from the extinction context. In the current experiments, we observed neither ABA nor ABC renewal. As mentioned previously, there are strain specific differences in the processing of contextual information [98-101, 126]. Inbreeding and transgenic manipulation of mice leave some strains with normal acquisition and extinction learning but show a definitive lack of sensitivity to the changing contexts. In our experiment, following the failure to observe renewal in the ABA arrangement, the decision was made to test a novel context with an extremely salient difference, namely the strong peanut odor previously used as a discriminative stimulus in our lab [68]. This alteration was still not enough to enhance the

perception of contextual cues for these subjects. In the Tat transgenic mouse line used in these studies, Tat is expressed using astrocytic based Tet-ON system that is temporally controlled by doxycycline administration [92]. There are several off-target effects using this method. First, doxycycline is an antibiotic that disrupts the gut microbiome [127]. The gut microbiome is important for a wide range of neural processes, including fear conditioning [128]. Specifically, disruption of the gut microbiome impairs AMG and HPC based functions during fear memory consolidation and recall as well as extinction learning [128, 129]. There is a need for more literature regarding the specific interaction between gut microbiota and context-based fear learning; however, given that HPC plasticity is disrupted following gut microbiota disruption, it follows that contextual information processing during fear conditioning is likely affected [129]. Secondly, astrocytes themselves exert direct influences on the fear conditioning process [130, 131]. Specifically, with regards to contextual memories, astrocyte activity in the HPC following training impairs contextual, but not cued, fear memory recall [130]. Therefore, lack of renewal could be explained by alteration of astrocyte function in these animals. Taken together, there is a clear justification to use Tat(-) subjects as control animals in these types of experiments, but this justification may also reveal why the transgenic line failed to show contextual sensitivities in these experiments. As such the inclusion of additional control groups examining the effects of the Tet-ON machinery independent of Dox treatment would be useful. Furthermore, the Tet-ON system in Tat transgenic mice is leaky causing low level astrocyte activation and inflammatory cytokine expression which may contribute to deficits independent of induced Tat expression [132].

In summary, these data revealed an acquisition and extinction deficits in male Tat(+) mice, but found no renewal effects in any of our subjects. The acquisition effects seem to be driven by disrupted intra-AMG activity, while the extinction deficits seem to stem from depressed sIPSC frequency in the PFC. The lack of renewal effect observed in these studies may call into question the appropriateness of this mouse model for renewal experiments. Further research using wild-type, or transgenic subjects without Dox treatment as control groups are needed to determine if the lack of renewal effect is due to strain specific features or Dox treatment.

CHAPTER 4: REINSTATEMENT

Reinstatement is a form of relapse that involves presenting the US alone following extinction. Notably in this arrangement, the CS is never presented during the reinstatement treatment, and as such the subsequent return of conditioned responding is due solely to the presentation of the US and not due to a strengthening of the CS→US relationship [1, 21]. Furthermore, while there are contextual effects with reinstatement when you depart from the reinstatement context, there are no such effects when the context is kept consistent throughout the experiment [1]. As such a study of reinstatement would look at an HPC independent form of relapse.

4.1 Reinstatement Circuitry

Regrettably, aversive conditioning reinstatement circuitry is not well studied at this time. Appetitive conditioning and reinstatement of drug seeking operant behaviors are more well understood; however these procedures engage reward, feeding, and motivation pathways that are not implicated in fear conditioning circuitry [7, 11].There is a great need for more research to be done investigating the exact mechanisms within these pathways for the reinstatement of conditioned fear. Nonetheless, the current understanding of reinstatement circuitry, as summarized in **Figure 7**, involves the AMG, IL, and ventral tegmental area (VTA).

The involvement of the AMG in reinstatement is thought to be primarily as it was in acquisition [9]. As previously stated, CS and US inputs converge in the BLA [16], the information is then projected to the CEA [89] where GABAergic neurons work on a system of disinhibition or feed outputs to motor control regions [17]. Notably, the projections from the BLA to the CEA are under the effects of neuromodulation from tightly packed inhibitory interneurons being innervated by the HPC and PFC [89]. During extinction, the IL innervates the inhibitory interneurons to gate the passage of information from the BLA to the CEA to prevent the production of a freezing response [12-15, 18]. It is thought that reinstatement involves a weakening of the IL's ability to innervate the inhibitory interneurons in the AMG [9]. In their study, Nomura and colleagues used c-Fos expression and electrophysiology to record activity in the IL following reinstatement treatment. From their results, subject who received a reinstatement treatment showed lower IL c-Fos expression, indicating reduced activity, compared to subjects who did not receive the reinstatement treatment. Consequently, researchers also noted increased c-Fos expression in the CEA of reinstated subjects compared to controls. This data was supported by electrophysiology data showing decreased miniature excitatory postsynaptic currents in the IL of subjects who received the reinstatement treatment compared to controls. From their understanding of dopamine dynamics in the PFC, this group of researchers hypothesized that dopamine type 1 (D1) signaling in the IL was the cause of reduced activity following reinstatement. They proposed that dopaminergic IL projecting VTA neurons were the source of this dopamine. In their experiment researchers used D1 receptor antagonists in the IL and PL to determine that halting dopaminergic activity specifically in the IL abolishes the reinstatement effect. Insights

from drug addiction literature reveal strong dopaminergic innervations of the PFC from the VTA are critical is producing flexible behaviors associated with transitioning drug states during addiction [8]. While the question, “why is the VTA involved” in associative learning requires further study, we know two relevant pieces of information about the VTA. First, the VTA codes for reward prediction errors [133]. A reward prediction error occurs when the actual value of a reward differs from the predicted value of a reward that has been signaled by some preceding cues. Applied to this circumstance, perhaps the role of the VTA in reinstatement is to code for the error between perceived safety from the US based on extinction training and the shock of getting the US during the reinstatement treatment. Perhaps this prediction error is extreme enough to suppress IL activity leading to the reinstatement effect. Secondly, the VTA receives inputs from the lateral habenula, and that VTA neurons which receive these inputs predominately project to the PFC [10]. In turn, the lateral habenula has been implicated in guiding fear and anxiety behaviors and have been shown to be activated in response to aversive stimuli [134]. Taken together, perhaps a pathway for reinstatement relies on the US exciting lateral habenula neurons projecting to the VTA which resolves the prediction error by inhibiting activity of the IL to prevent the inhibition of BLA→CEA activity. In other words, for our current project, inactivity in the IL results in reinstatement.

4.2 Current Project

Given that IL suppression plays a critical role producing the reinstatement effect, and disruption/failure of IL suppression prevents the reinstatement effect, it follows that in cases where the IL is already failing to inhibit fear response during extinction that reinstatement should not be affected [9]. As such Tat-induced deficits in IL excitation

should not prevent the reinstatement effect from occurring [64, 66]. In the current project, we therefore hypothesized that Tat would not affect modulations of IL activity during reinstatement and as such we should observe unaffected reinstatement effects in our Tat transgenic mice. Rather than revealing a deficit, this experiment served to demonstrate that any renewal deficits are not due to a failure to recall the acquisition memories following extinction as reinstatement to the CS should only occur if the acquisition memory remains intact.

4.3 Subjects

Subjects were used as previously described in section 2.3. In this experiment, there were 8 subjects per group.

4.4 Apparatus

These experiments used the same apparatus as previously described in section 2.4. All training and testing sessions took place in the Context A arrangement (darkened chamber) described in section 3.5.

4.5 Procedure

Acquisition and extinction proceeded as previously described in sections 2.5 and 3.6 respectively. Following the final day of extinction, a reinstatement treatment was provided in which a single unsignaled presentation of the US occurred following a 3 min habituation period. The next day, testing occurred by presenting the CS in the absence of the US after 3 min.

4.6 Statistical Analysis

Statistical analysis proceeded as previously described in sections 2.6 and 3.7.

4.7 Results

Reinstatement is a procedure where the subjects are reintroduced to the US following extinction. As previously mentioned, reinstatement is a largely HPC independent process; thus, we hypothesized that the Tat protein would largely leave this process undisturbed owing to the extensive research showing hippocampal deficits associated with the presence of Tat. Unlike the renewal experiments, this experiment did not alter the context and used the "Context A" arrangement previously mentioned for every phase. Acquisition and extinction proceeded as previously described (n = 8/group); however, 24 h following the final day of extinction, subjects received a reinstatement treatment before proceeding to testing 24 h following this treatment. As discussed previously, reinstatement involves unsignaled US presentations and as such any relapse in the CR is not due to new learning about the CS→US association. The data for this experiment is summarized in **Figure 8**.

Much like the ABC Renewal experiment, all subjects demonstrated normal acquisition and extinction, although there were no significant differences between any of our groups (all p's > .05). The reinstatement treatment was a success and resulted in a return of the conditioned response; however, there were no significant differences between any of our groups, p > .05. Taken together this experiment results in two meaningful conclusions. First, the Tat protein does not seem to affect reinstatement. Second, the Tat transgenic line is capable of remembering even single associations after

ten days. Taken together, we can draw the conclusions that since the Tat transgenic mouse is capable of relapse over moderate delays, but did not show relapse effects, then the machinery for context independent forms of relapse are largely intact while the machinery for contextual processing in the Tat transgenic mouse (both Tat(+) and Tat(-)) is impaired.

4.8 Discussion

The reinstatement experiment was designed to test relapse in the CR following extinction in a way that is largely independent of the HPC. In contrast to the renewal studies, which heavily depend on the HPC, these data show a robust reinstatement effect following extinction that is consistent with previous literature's findings on how Tat affects structures within the reinstatement circuitry. Reinstatement occurs due to suppression of IL inhibition of the BLA→CEA pathway [12-15, 18]. Previous work from our lab has shown inhibitory signalling is disrupted within the PFC [64, 66]. Additionally this work shows that the disrupted GABAergic activity in the PFC has behavioral consequences where Tat(+) mice showed poorer inhibitory control over operant behaviors [64]. In the current study, this disruption of GABAergic activity in the IL would account for the reinstatement effect observed; however, another explanation that would also produce the reinstatement effect is that everything was functioning as intended. If one were to entertain that IL GABAergic activity was disrupted, then this begs the question, why wasn't extinction affected? HAND is a slow moving, progressive, neurodegenerative disorder that often affects individuals in very subtle ways [27]. The length of induced Tat expression prior to testing affects the severity of observed deficits [92, 135]. In the current study, Tat expression was only induced for 2 weeks prior to the beginning of the experiment and as such may have not

had time to fully disrupt IL functioning to the point where extinction was affected. Concurrently, it would follow that salient even such as the reinstatement treatment would be proximal and intense enough to trigger VTA supression of IL function for the reinstatement test [9].

Indeed this experiment was not designed to answer such questions, instead this experiment served as confirmation that long-term memory of acqusition was not disrupted over the course of the experiment and as such relapse is possible in the Tat transgenic mouse. If the subject was unable to recall the acqusition memory following extinction as a result of Tat expression, then this would have obscured conclusions about the lack of renewal effect in those animals. As it stands, renewal did not occur for any of our subjects and from this study we can conclude that the lack of renewal was not due to a lack of recall about the CS→US association.

CHAPTER 5: GENERAL DISCUSSION

This series of experiments were designed to test behavioral deficits in the HIV-1 Tat transgenic mouse model of HAND as a way to index specific failures with associative learning. Using fear conditioning methodology, this series of experiments investigated acquisition, extinction, and relapse deficits. In two of the five studies examining acquisition, there were clear deficits of the female Tat transgenic mice in one experiment, and of the male Tat transgenic mice in another. While these individual results were not consistent throughout the series, the discussions for these individual cases highlight potential environmental causes that may have aided with the detection of these differences. Indeed within the literature as well, the usage of the Tat transgenic mouse model has seemingly produced contradictory behavioral results between individual studies (i.e. [71, 72] or [130, 136, 137]). It appears that the detection of behavioral deficits is dependent on a number of different factors, including level of Tat expression, sex-dependent CR phenotypes, and type of task chosen. Chiefly among these factors is both the length and method of inducing Tat expression in transgenic mice. While subjects in this study were fed Dox infused chow to activate the Tet-ON system, another method of inducing Tat is through acute injections [138]. One previous experiment that focused on induction method and length of Tat expression found that subjects receiving injections of Dox showed greater hippocampal related deficits than subjects that had been induced via Dox infused chow when length of induction remained constant [138]. Additionally, as one may expect, using either induction method over a prolonged period of time revealed

deficits that were not found during shorter periods of induction [138]. Likewise, similar studies that controlled for both dose and duration dependent effects of Dox injections found similar result in that hippocampal related behavioral deficits only appeared for higher doses of Dox [139]. Furthermore, these researchers found that lower levels of Dox administration (100mg/kg for 14 days) produced transient increases in anxiety behaviors with a high degree of variability within groups while a slightly higher dose (125mg/kg of Dox for 7 days) produced consistent elevated anxiety [139]. Of note, the dosage used in the current experiments was a low dose 6 mg/g Dox infused in chow for 14 days prior to and during the experiments which may explain our own transient findings. While this may at first glance seem like a weakness of the current experiments, it is in fact a more ecologically valid method for inducing Tat expression that mirrors the more gradual, but progressive, neurodegeneration seen in HIV infected humans [25-28, 92]. In humans the degree of neurocognitive impairments are directly correlated to the success of cART therapies and as such a large proportion of the HIV infected population never proceed past the ANI stage of HAND progression [27]. In this experiment, it is possible that individual subjects were differentially impacted by the low dose Dox administration and thus were not impaired to the level one would need to observe stark deficits in associative learning. Perhaps lengthening the period of Dox treatment may reveal deficits that had not yet fully manifested.

An interesting manipulation to the current methodology would be the addition of groups with longer periods of Dox treatment. We know that the intensity of certain deficits are dependent on the length of Tat expression prior to testing [92, 135] and as such one would expect greater deficits for longer periods of Dox treatments. Given that fear

conditioning research is extremely vulnerable to environmental confounds (i.e [104, 110]), it would be prudent not to start all of the animals simultaneously on Dox treatment. For instance, if subjects were meant to be tested after 2, 4, and 6 weeks of treatment, then it would be unwise to test all of the 2-week animals together. Then all the 4-week animals. Then all the 6-week animals. Suppose something happened during the week 6 test that did not occur during the week 2 test? Instead all subjects require staggered start times so that on any given test day all groups are represented. This design could be implemented fairly straightforwardly in future experiments if one were so inclined. To address this issue, further studies could also employ a single subject design in which each subject serves as a replication of previous subjects rather than an aggregated group [140].

A single subject dosing would allow a more focused look at how individual subject differences in Tat expression, immune responses, dendritic morphology, etc. may contribute to behavioral deficits. Additionally it allows better control of error variance in behavioral data due to environmental factors [141]. Note that in aggregated group designs for fear conditioning, confounding variables are accounted for by testing all subjects under the same conditions at the same time. Thus, any confounding variable affects all groups equally. This method is extremely robust for examining results within a study, but significantly weakens comparisons between experiments. For instance, the individual discussions within this document describe cases where changes in time of day, housing conditions, and other environmental variables may have altered the pattern of deficits observed in the subjects between studies. One major weakness of the single subject design approach would be it's typical use for detection of clinically relevant effects

on behavioral data [141]. For a subtle disorder like HAND, this design may lack the power to observe the small effect size of Tat induced deficits [27]. Beyond using different experimental designs, it may be useful to group subjects not by their genetic background, but by their levels of Tat expression. In other words, group animals as no, low, or high levels of Tat expression and correlate these data with the observed deficits. While it may seem an obvious grouping, a method for the accurate and reliable quantification of CNS Tat protein remains elusive due to poor sensitivity of antibody and lipid-based methodology.

As evident in the current series of experiments, sex is an important factor for the observed deficits. In the present series of experiments, males and females showed acquisition deficits, though never at the same time, and only males showed a transient extinction deficit concurrent with an acquisition deficit. Within the fear conditioning literature, there are sex differences in phenotypic fear responses [142, 143]. Depending on the type of stimuli presented, rodents engage in both active and passive fear responses dubbed “darting” and “freezing” [142]. Before the inclusion of female subjects in behavioral research, darting was thought to be burst of locomotor activity thought to be brought about by imminent contact with a predator; meanwhile, a freezing response is a suppression of behavior brought about by the threat or proximity of a predator [144]. In other words, darting occurs to the US while freezing occurs to the CS; however, depending on the arrangement of stimuli in an experiment this may not always be the case [142, 145]. Instead, some subjects, typically but not always females, display freezing behaviors to the initial CS presentation, but transition to darting behavior near the tail end of a stimulus [142, 145]. Additionally in higher order conditioning studies,

freezing occurs to stimuli temporally distant from the US, while more proximate stimuli show more darting behaviors in primarily female mice [145]; although some more recent data indicate stimulus salience, rather than temporal proximity to the US, is the factor which governs active or passive responses [146]. Moreover, while previously categorized as a post encounter defense related more to panic than a CR [147], these active CR's acquire and extinguish the same as passive CRs [142, 143, 145]. Importantly, active and passive fear responses are the result of a competitive inhibitory process within the CEA [145]. In other words, the selection of an active fear response is mutually exclusive with a passive response, and once a subject has selected an active or passive phenotype, it will stick with that response for the entire experiment [142, 143, 145]. With regards to the current series of experiments, by only using one method for detecting fear, it is possible we have ignored an important manifestation of the CR, especially in our female subjects. To account for this, future experiments would need to use a different method to assess fear.

Perhaps the easiest alteration to the current methodology would be to simply use locomotor activity as a measurement of darting [142]. Locomotor activity during the CS would serve as a grouping variable to separate the two phenotypes. Another method uses the looming predator task which has gained recent popularity and is sensitive to topographic variations in the CR [148]. This task uses video screens affixed to the roof of an open chamber with a small escape tent-like structure situated in one of the corners. The video screen animates a black circle in either a sweeping pattern, which moves the circle back and forth across the screen, or a swooping pattern, which moves the circle to the center then rapidly enlarges it. In the case of the swooping stimulus, there is a near

total activation of active fear responses, while the sweeping stimulus predominantly activates passive fear responses (the proportion of which can be altered further by sweep speed) [148]. Thus, depending on the stimulus used, one could independently elicit and manipulate active and passive fear responses. As an extra bonus, these stimuli are aversive US's that completely minimize the pain to the subject. This task is however, not without its weaknesses. Current efforts to pair NS with the visual US have not been fruitful [149]. Ideally, since the interest of these experiments is in associative learning, it would be best to use a task the induces learning in our subjects and while one study cites acquisition failures, more experiments are needed to determine whether subjects can learn in the looming predator task. As there have not been any studies showing successful acquisition yet, no one has tried extinction using these methods. Critical to extinction is experiencing the CS in the absence of the US. Depending on the CS chosen, this apparatus allows the subject to escape from the testing area into the small tent structure. If for instance a visual stimulus was chosen, then there is a possibility for the animal to simply avoid the stimulus and consequently avoid extinction. Would failure to extinguish indicate deficits or an extremely effective coping strategy? One solution would be to block the escape route, but would this modify darting behaviors? On a related note, one requirement of this task is that the subject actually move underneath the screen to trigger the US and ensure detection from the subject [148]. Thus, in a sense, the US also as a conditioned punisher that lowers the probability of the mouse venturing to the center of the field. So again, if subjects have a route to escape, they may instead exhibit a learned avoidance response as seen in predator odor experiments [150, 151]. From a data analysis perspective, would any deficits regarding acquisition and extinction simply

be a finely tuned avoidance response? From a methodological perspective, would the variations in chamber habituation lengths due to variability in mouse ambulation alter contextual effects such as renewal or contextual fear conditioning? The procedure would also poorly control for environmental confounds associated with variability in US timing. Despite these weaknesses, if methodology for establishing a learned association using this task is established, the looming predator task would be an excellent way to parse freezers and darters and categorize Tat's effects on these two competing CRs.

5.2 Future Studies

The transient acquisition and extinction deficits observed in the current project reveal dysfunction within intra-AMG circuitry as well as connections between the IL of the PFC. As a reminder, the acquisition circuit is primarily localized in the AMG. CEA projecting BLA neurons integrate sensory information about the CS and US [16, 17]. In the CEA, tightly interwoven excitatory and inhibitory neurons engage in a competitive process which results in the production of the CR [17, 84-87]. Reciprocal AMG to PFC, PFC to HPC, and HPC to AMG connections code for contextual information important for acquisition and synchronize firing between trial relevant neuron subpopulations in the HPC and AMG [17, 19, 20]. As a result of exitncion, the IL inihibits activity within the CEA to prevent the expression of the CR [12-15, 18]. The success of extinction treatments is directly dependent on the inhibitory tone of the IL at retreival [18]. The current series of experiments saw cued, but not contextual, fear conditioning deficits indicating that the AMG is affected by Tat expression. Additionally the renewal effect was not observed in any subjects. As such, future experiments down this line should primarily seek to investigate the specific effects Tat has on the AMG and PFC.

Given that the expression of the CR is a competitive process between inhibitory and excitatory neuronal populations, investgating whether the balance between inhibitory and excitatory synaptic proteins are disregulated may provide clues to the effects of Tat on this system. One change that has been attributed to the deficits observed in the acquisition one trial contextual fear conditioning is disruptions to the balance of inhibitory and excitatory synapses [71]. Within the HPC previous data shows decreases in the inhibitory presynaptic protein Synaptotagmin 2 (Syt2) and increases in the postsynaptic inhibitory protein Gephyrin [71, 152]. Furthermore, *in vitro* data have shown the excitatory postsynaptic protein PSD-95 to be disrupted by Tat [152, 153], while *in vivo* data have shown this protein to be unaffected [71, 154]. Within the PFC, Tat has also been shown to disrupt inhibitory synapses by decreasing both Syt2 and Gephyrin while simultaneously increasing Gad67 [153]. The disruptions to inhibitory circuitry either by upregulating or downregulating the amount of GABAergic connections in these different regions carry behavioral significance and warrant further investigation; however, there has been no analysis to date of Tat's effects on excitatory and inhibitory synaptic protein expression in the AMG. Importantly, disruptions to the balance of inhibitory and excitatory synapses have been shown to have behavioral consequences for behaviors associated with these structures [71, 153, 154]. To investigate synaptic integrity and balance in all these regions, western blot protein analyses investigating Tat's effects on excitatory presynaptic proteins Synaptotagmin 1 (Syt1) and Postsynaptic density 95 (PSD-95), as well as excitatory postsynaptic proteins GAD 67, GluR1, and NMDAR2A could be employed. Additionally the analysis could look at Tat's effects on inhibitory presynaptic protein Syt2 and postsynaptic proteins Gephryin and Gamma-aminobutyric acid receptor subunit

alpha-1 (GABAAR α1). Some of these proteins, such as Gephryin, PSD-95, Syt2, and Glutamic acid decarboxylase 67 (GAD 67), have been found to be altered during the course of Tat expression [71, 153, 154] and have been attributed to deficits in behaviors associated with structures where these alterations have been found. Observing the impact of the HIV-1 Tat protein on these proteins will give insight into where and how Tat exerts its effects.

Tat is also known to affect the dendritic morphology of affected neurons, and the severity of synaptic disruption and dendritic injury is correlated with the severity of expressed symptoms of HAND [41, 42]. As such, another experiment could look at changes to neuron structure. One structural change Tat has on neurons is reduction in dendritic spine densities which lead to behaviorally significant outcomes [155-157]. Future studies could pair Golgi staining with behavioral data to investigate the impact changes to dendritic spine density within the AMG and PFC have on acquisition and extinction [158]. While this method would not be specific to a particular cell type, the use of green fluorescent protein (GFP) attached to PSD-95 and Gephryin promoters could target the structure of specific types of synapses and investigate whether they are being differentially affected by Tat expression [159, 160]. PSD-95 in particular has been shown to be a key regulator for determining the ratio of excitatory and inhibitory synapses [161]. One limitation of this approach would be the historical lack of dendritic spines on most inhibitory interneuron populations [162]; however, recent advances in electron microscopy have shown evidence for spines on disinhibitory interneuron populations implicated in associative learning [163]. Additionally, it has been found that spines on these interneurons are extremely dynamic and typically have shorter lives than spines on

pyramidal neurons [163]. Therefore, given the critical role these neuron populations have on gating CR's within the AMG, this limitation would need to be supplemented by additional methodology [89].

In keeping with the theme of inhibitory and excitatory synapse balance, the function of these synapses is also affected by Tat [64, 164]. In addition to examining the structural changes caused by Tat future studies could examine changes to miniature and spontaneous, excitatory and inhibitory, post synaptic currents (EPSC and IPSC respectively). There are region specific changes in neuronal excitability as a result of Tat [165]. In the PFC, previous research has shown that EPSC frequencies are increased following Tat expression, while IPSC frequencies are reduced leading to an overall increase in the excitability of PFC neurons [64, 164, 165]. In contrast in the HPC, the effects of Tat tend to lean toward increasing the inhibitory tone [165, 166]. While the HPC and PFC have been extensively studied, the functional changes to the AMG have received far less attention. Given the increased excitability of the PFC under Tat expression, it stands to reason that any disruptions to extinction are likely due to a failure of the IL to send inhibitory signals to the interneurons governing BLA and CEA connections. As such the ability to correlate observed deficits with overall excitability of the PFC would draw direct connections between Tat's effects and observed behavioral deficits. Additionally, any data looking at electrophysiological changes due to Tat expression would be as novel as they are informative.

5.3 Conclusion

The current series of experiment found transient deficits in acquisition and extinction in the Tat transgenic mice. These findings were sex dependent with males and females independently showing acquisition deficits while only male Tat(+) animals displayed extinction deficits. These findings offer a strong foundation for further research into the biological factors underlying Tat's damage to the system as well as warrant a closer look at comparisons for length of Tat expression. Furthermore, these experiments saw no renewal effect in any subjects, possibly due strain specific insensitivity to contextual information, but did see reinstatement effects indicating lack of renewal was not due to failure to recall the CS→US association. While the lack of renewal in control subjects was unfortunate, further research into specific changes the Tat Tet-ON system makes to transgenic animals could implicate important proteins to renewal circuitry.

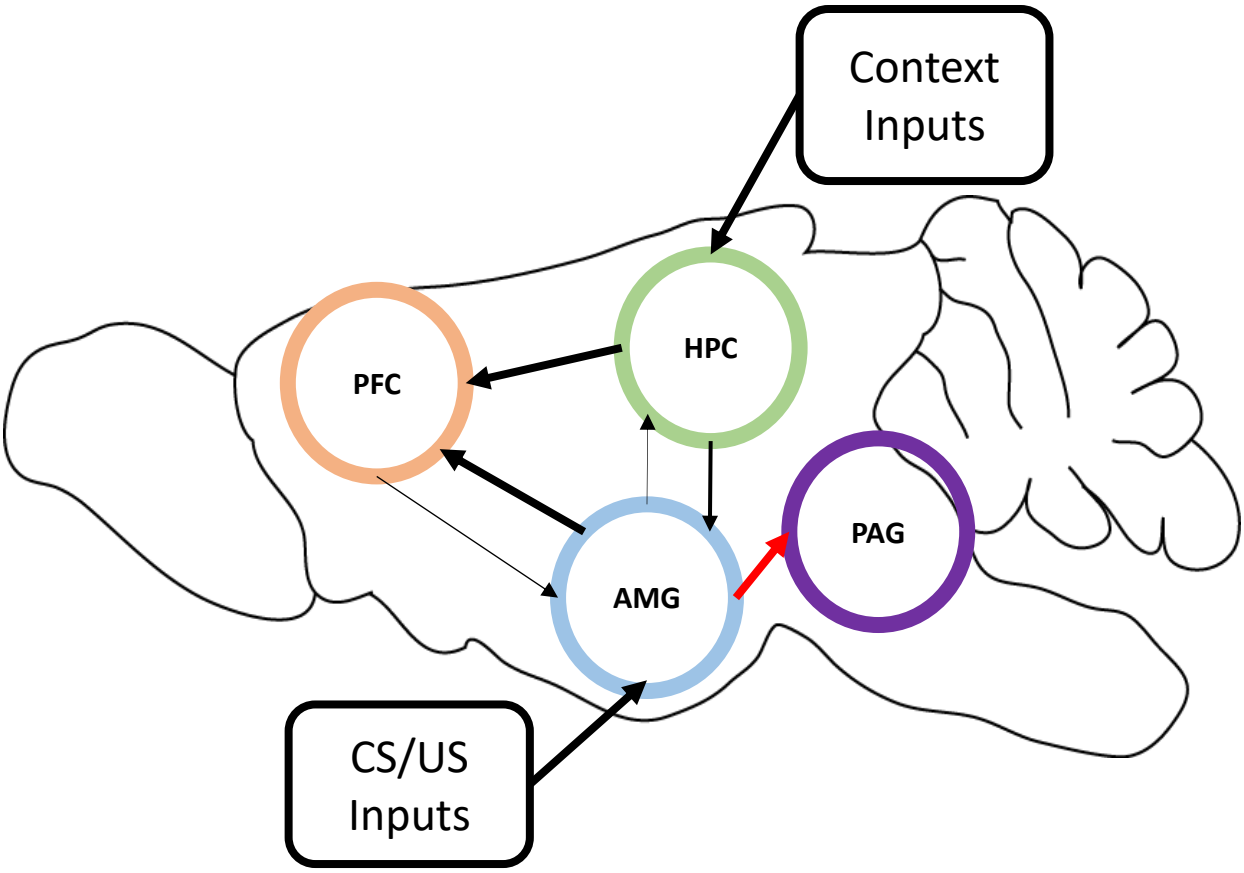

Figure 1. Acquisition Diagram. This diagram summarizes the circuitry within acquisition, notably black lines indicate a net excitation while red lines indicate a net inhibition. The thickness of the lines corresponds to the overall level of innervation occurring between regions. Acquisition is largely considered an intra-AMG process in which communication between the BLA and CEA of the AMG determine the intensity of CR [16]. Within the BLA converging CS and US information innervate glutamatergic neurons which project to the CEA where tightly woven excitatory neurons and inhibitory neurons within these regions participate in a competitive process. Freezing, our measurement of fear, occurs when the result of this competition favors excitation in the CEA which results in inhibition of the PAG [13, 17, 18]. The AMG is subject to influences from the HPC, which provides contextual information, and the PFC which helps gate the connection between the BLA and CEA [4, 6, 19]. While this diagram represents cued fear conditioning, in a contextual fear conditioning study the CS information converging in the BLA would be fed forward by the HPC and thus the connection between the HPC and BLA would see greater activity [20].

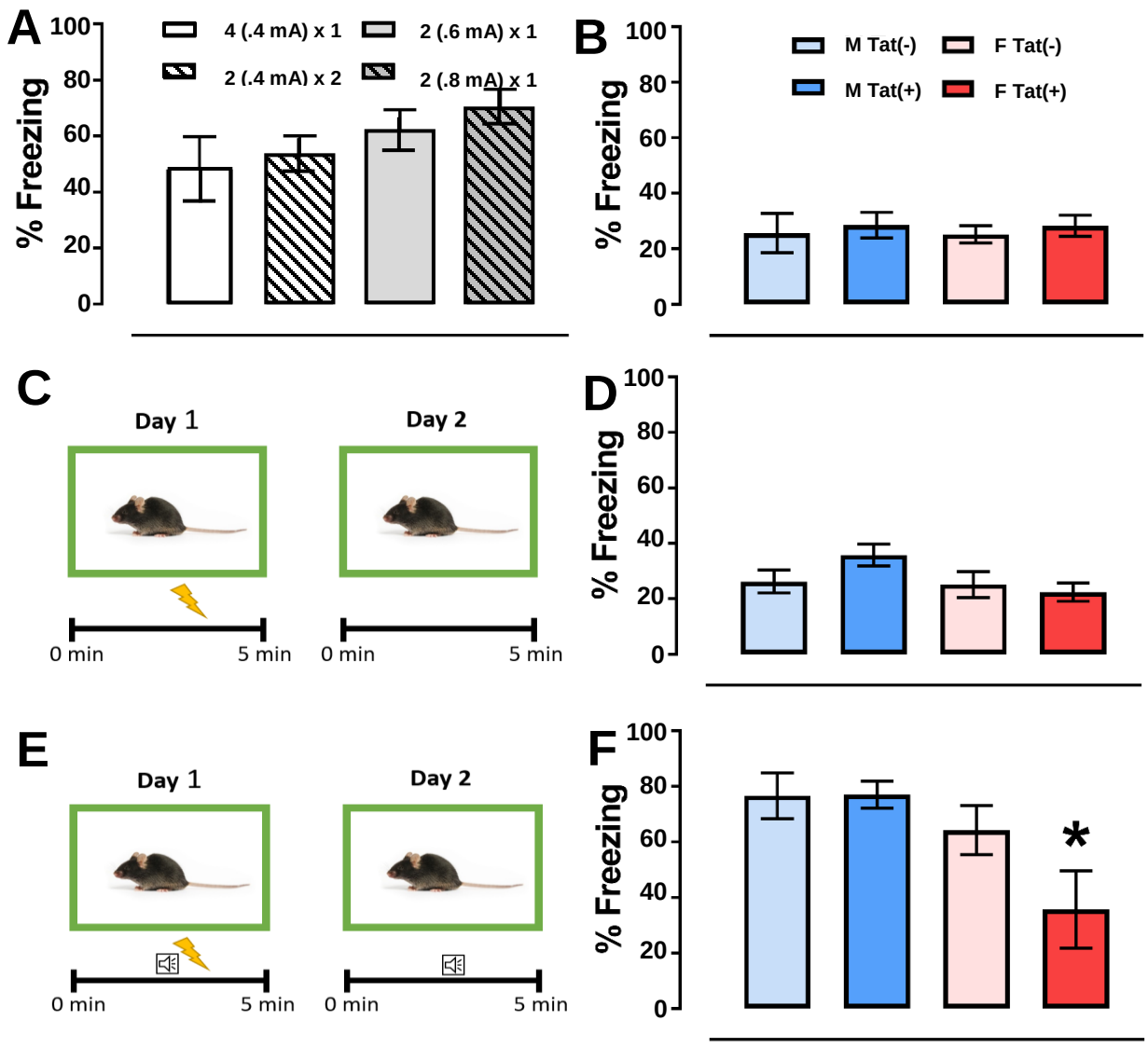

Figure 2. Shock Titration and Acquisition

Figure 2. Shock Titration and Acquisition: Taken together this data demonstrates a clear acquisition deficit in Tat(+) female mice undergoing single trial cued fear conditioning and a lack of support for contextual fear conditioning deficits in Tat transgenic mice. **(A)** Subjects were exposed to either four .4 mA shocks on one day, two .4 mA shocks on two days, two .6 mA shocks on one day, or two .8mA shocks on one day. This data shows the .4 mA shock presented 4 times on one day was effective for conditioning a fear response while minimizing distress to the subjects. **(B)** Using the .4 mA US presented 4 times on a single day, subjects underwent a contextual freezing paradigm in which the context served as the CS. Freezing behavior was measured 24 h following acquisition across the entire 5 min session and no significant differences were found between any of the groups (all $p > .05$). **(C/D)** In a separate study, subjects underwent a contextual freezing paradigm which used a single, more intense (.7 mA) US. As before, there were no significant differences in total amount of freezing between any of our groups. **(E/F)** Subjects underwent a cued fear conditioning paradigm which paired a 60 s 2000 Hz tone CS with a single .7 mA US. There was a main effect of sex on freezing behavior during the CS, $p = .007$. *: Bonferroni post hoc tests revealed that female Tat(+) animals exhibited significantly less freezing female Tat(+) mice froze less ($M = .389$, $SEM = .161$) than male Tat(+) mice ($M = .798$, $SEM = .048$), $p = .029$, and male Tat(-) mice ($M = .767$,$SEM = .083$), $p = .021$. These results indicated that when presented with a single presentation of the US, Tat(+) female mice froze significantly less than any other group (*) indicated a failure to encode fear memories associated with the 60 s cue. mA = milliamps

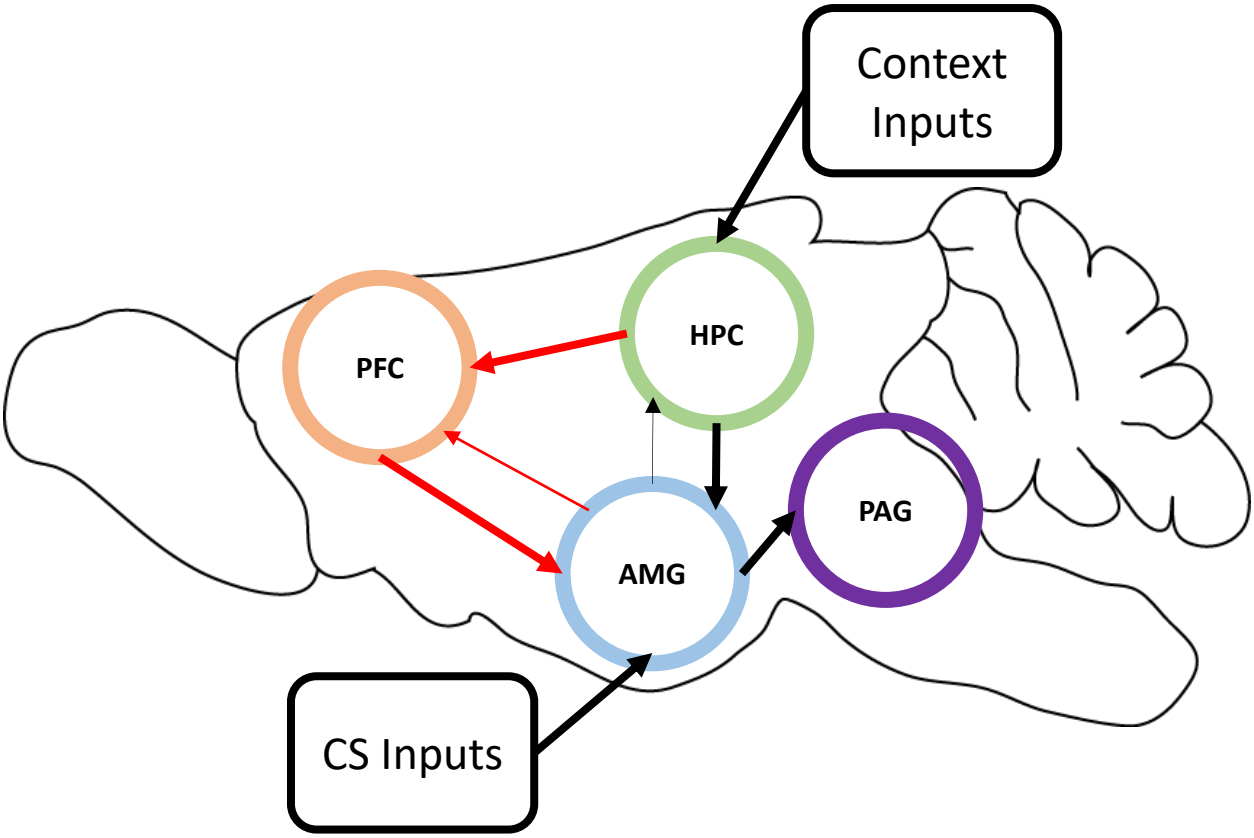

Figure 3. Extinction Diagram. This diagram summarizes the circuitry within extinction. The diagram is organized as previously described in **Figure 1**. Extinction is largely thought to be under the control of IL activity within the PFC. [15]. The IL contains predominately GABAergic neurons which innervate inhibitory interneurons gating the BLA→CEA pathway active during acquisition [12-14]. This results in a failure to disinhibit these connective pathways which halts the inhibition of the PAG thus preventing the freezing response [15]. Importantly, this activity does not erase the innervation of the CEA from the BLA seen in acquisition, but rather interferes with this process [21]. Extinction is highly context specific, and as such the role of the HPC in conveying contextual information is much more active [5, 6, 22, 23]. Despite the critical role the HPC plays, it is not completely necessary during the extinction process [23, 24]. Reciprocal connections between the IL, HPC, and AMG help to synchronize firing of neurons within this circuit under appropriate stimulus conditions [22].

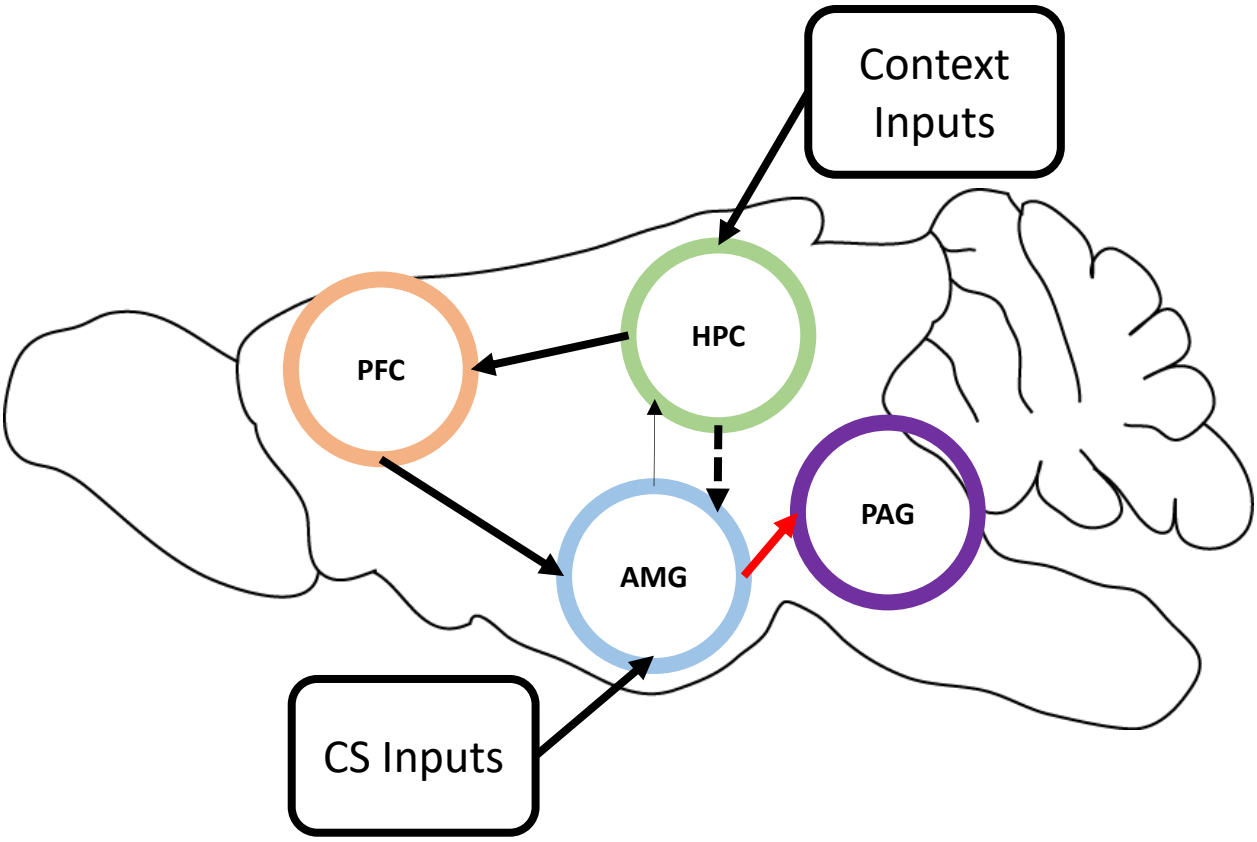

Figure 4. Renewal Diagram. This diagram summarizes the circuitry within renewal. The diagram is organized as previously described in **Figure 1**. Direct and indirect connections between the HPC and the AMG underlie the renewal effect [1-6]. The direct pathway takes contextual information form the HPC to the BLA. When the direct connection between the HPC and AMG is disrupted, the renewal effect is lost; however, this pathway sees far less activity during renewal than the indirect pathway [3, 4]. A second pathway routes contextual information from the HPC to the PL of the PFC. From there the PFC innervates the BLA of the AMG to produce the freezing effect as previously described [3, 4, 6]. Inactivation of this pathway also prevents the renewal effect from occurring [3, 4, 6]. It's thought that the PL synchronizes firing between the HPC and AMG neurons resulting in the relapse of conditioned responding [6].

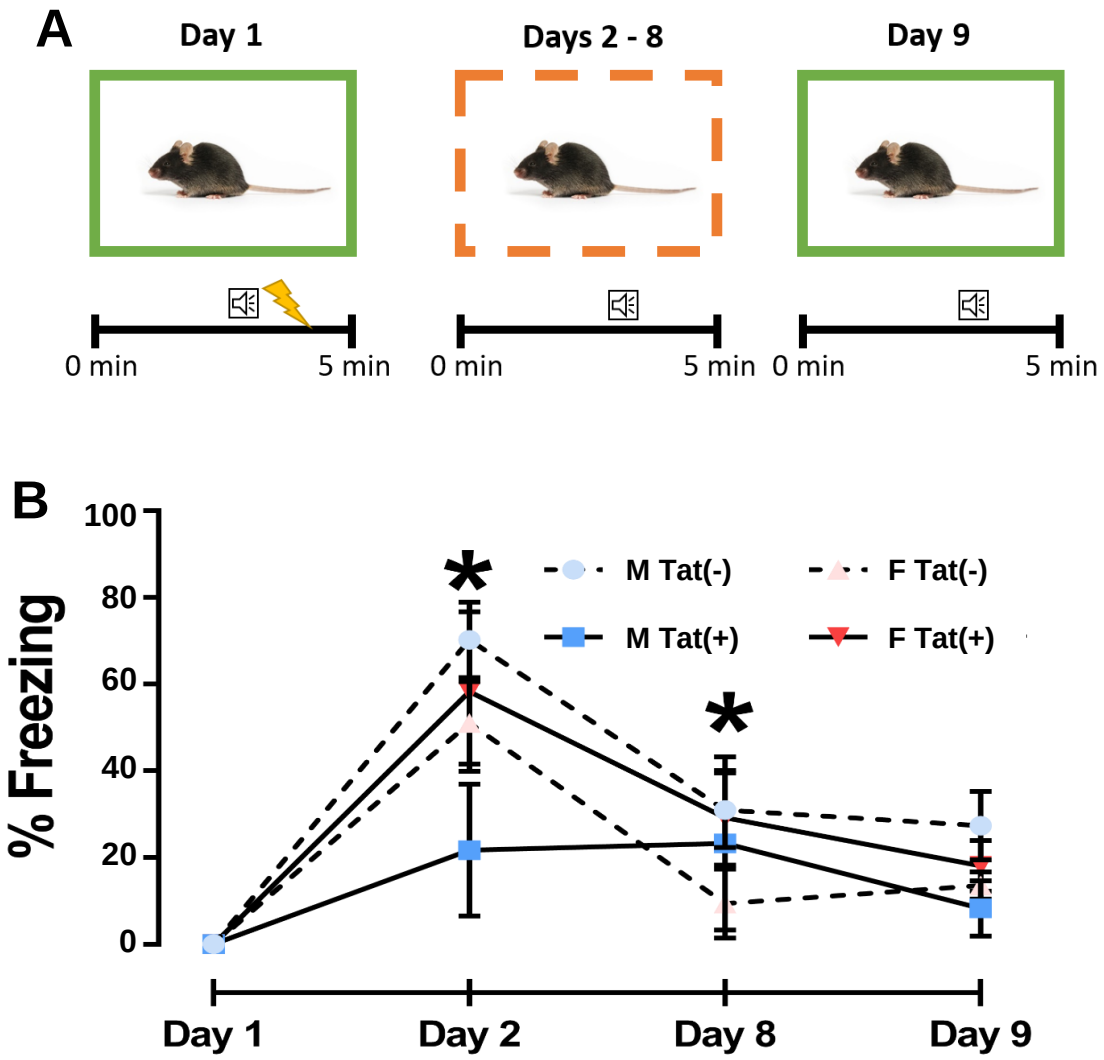

Figure 5. ABA Renewal: Male Tat(+) subjects show transient acquisition and extinction deficits while no subjects show renewal. **(A)** Fear was acquired using the cued fear acquisition paradigm previously described in context A before undergoing 7 days of extinction training in context B in which the 60 s CS was presented in the absence of the US. After cessation of extinction training, subjects were returned to the original acquisition context and presented with CS in the absence of the US. **(B)** Data shown is from the first day of acquisition (Day 1), the first day of extinction (Day 2), the final day of extinction (Day 8), and the renewal test (Day 9). Acquisition deficits are determined based on Day 2 data, while extinction deficits are determined based on the difference within group between Days 2 and 8. Likewise, renewal effects are determined by the differences within group between Days 8 and 9. There was a main effect of genotype on acquisition learning p = .038, and extinction learning, p = .045. There were no significant main effects for the renewal test, p > .05. Despite the lack of significance in the Bonferroni post hoc tests, these results show that Tat expression results in both acquisition and extinction deficits.

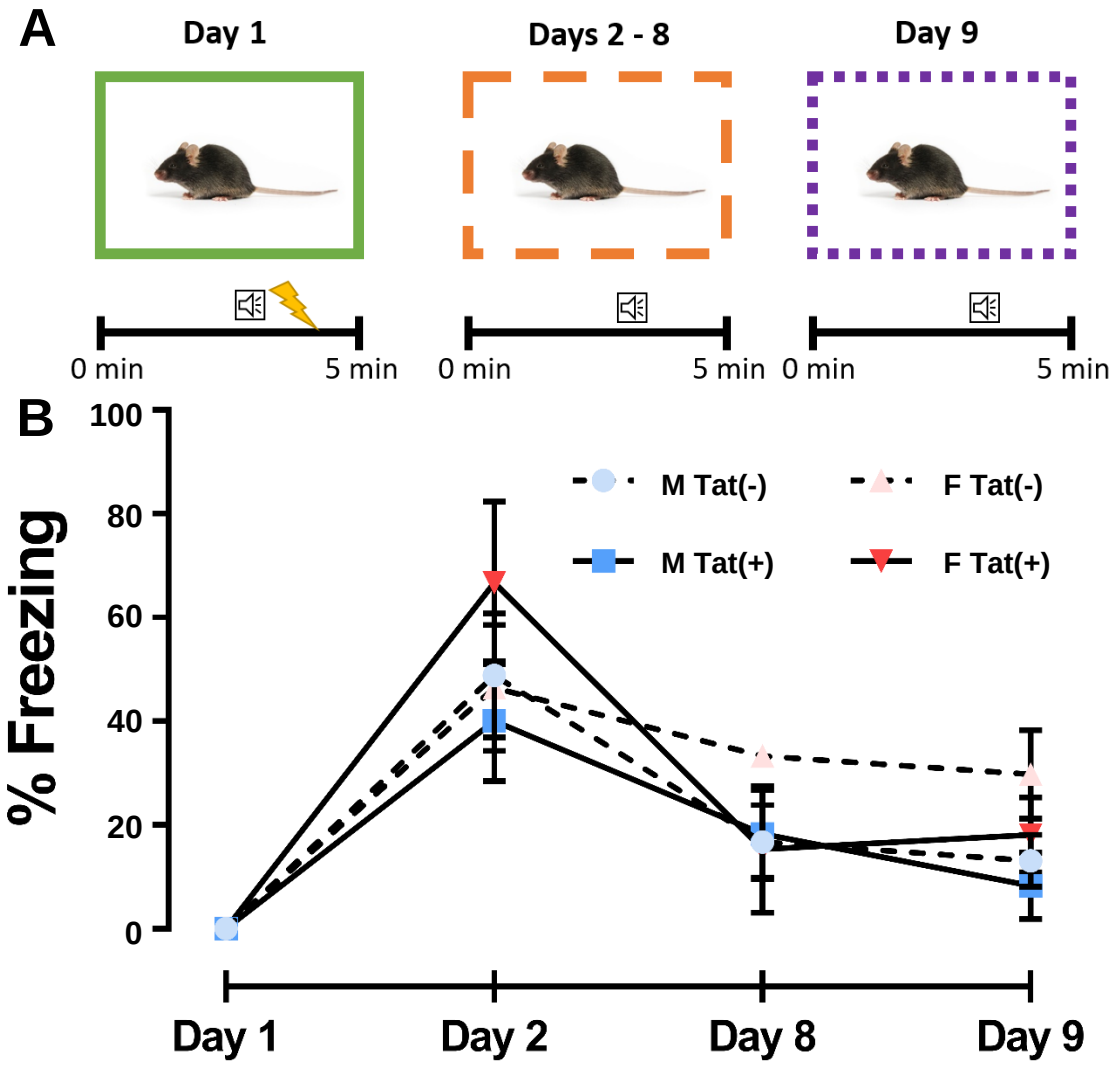

Figure 6. ABC Renewal: All subjects show typical acquisition and extinction but fail to show renewal effects even when the testing context is made more salient. **(A)** Fear acquisition and extinction proceeded the same as previously described. For the renewal test, subjects were moved to a novel context consisting of a unique olfactory stimulus and presented with CS in the absence of the US. **(B)** Data is presented in the same manner as in **Figure 5**. There were no significant differences between any groups (all $p > .05$). Taken together with data from **Figure 5**, these two data sets demonstrate a lack of contextual sensitivities during fear conditioning procedures independent of Tat expression.

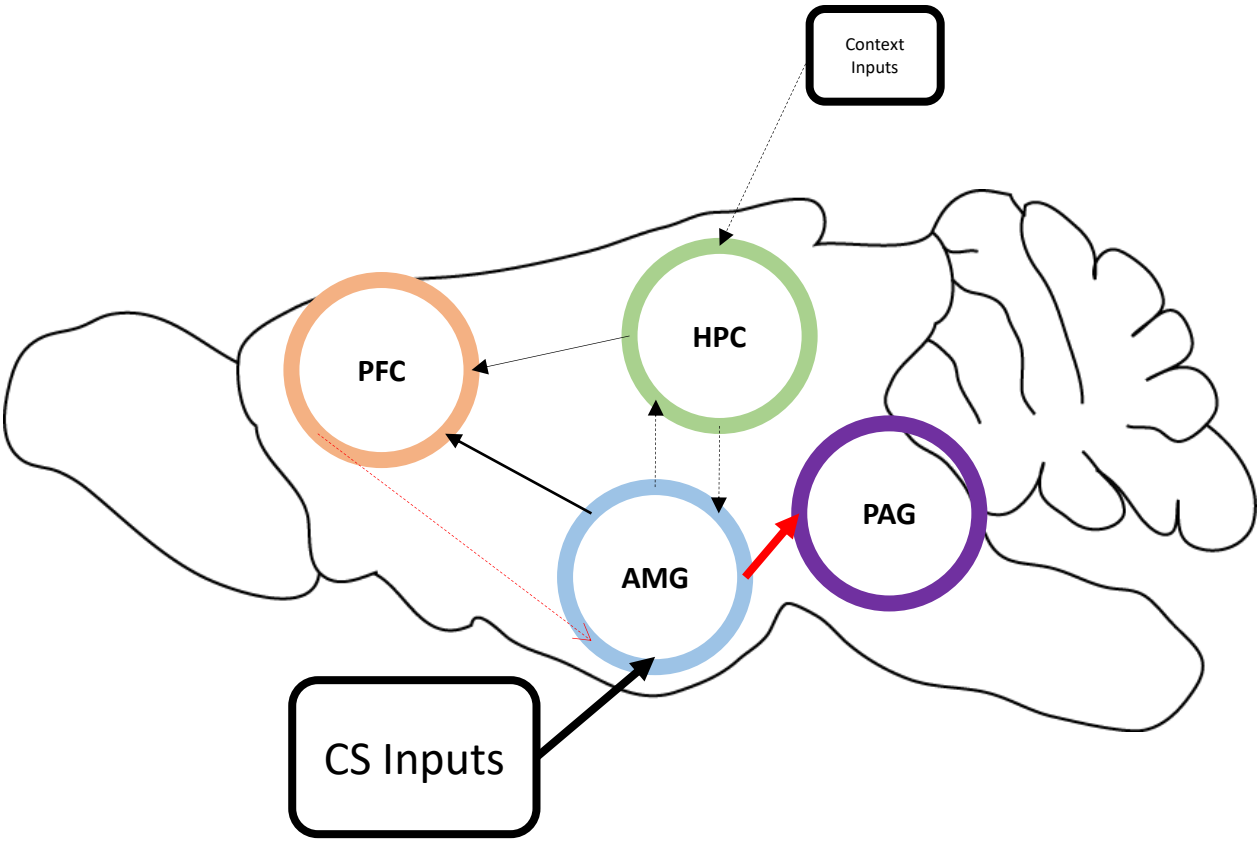

Figure 7. Reinstatement Diagram. This diagram summarizes the circuitry within renewal. The diagram is organized as previously described in **Figure 1**. There is a need for far more research into the circuitry for reinstatement of fear memories; however, current understanding of reinstatement points towards disruption of IL inhibition of intra-AMG circuitry as the key player in this form of relapse [7-11]. The performance of the CR following reinstatement is due to competing inhibitory and disinhibitory processes in the AMG [9-11]. In extinction, increased IL activity is responsible for inhibiting activities in the AMG thus halting freezing [12-14]. Studies of fear reinstatement show DECREASED c-Fos dependent activity in the IL for groups that receive reinstatement treatments [9]. Additionally, Reinstatement treatment results in reduced mEPSC frequency, a pattern similar to groups that did not receive extinction [11]. One proposed mechanism the VTA dopaminergic activity is depressing the IL, although there is a need for further research as to the exact mechanisms [9].

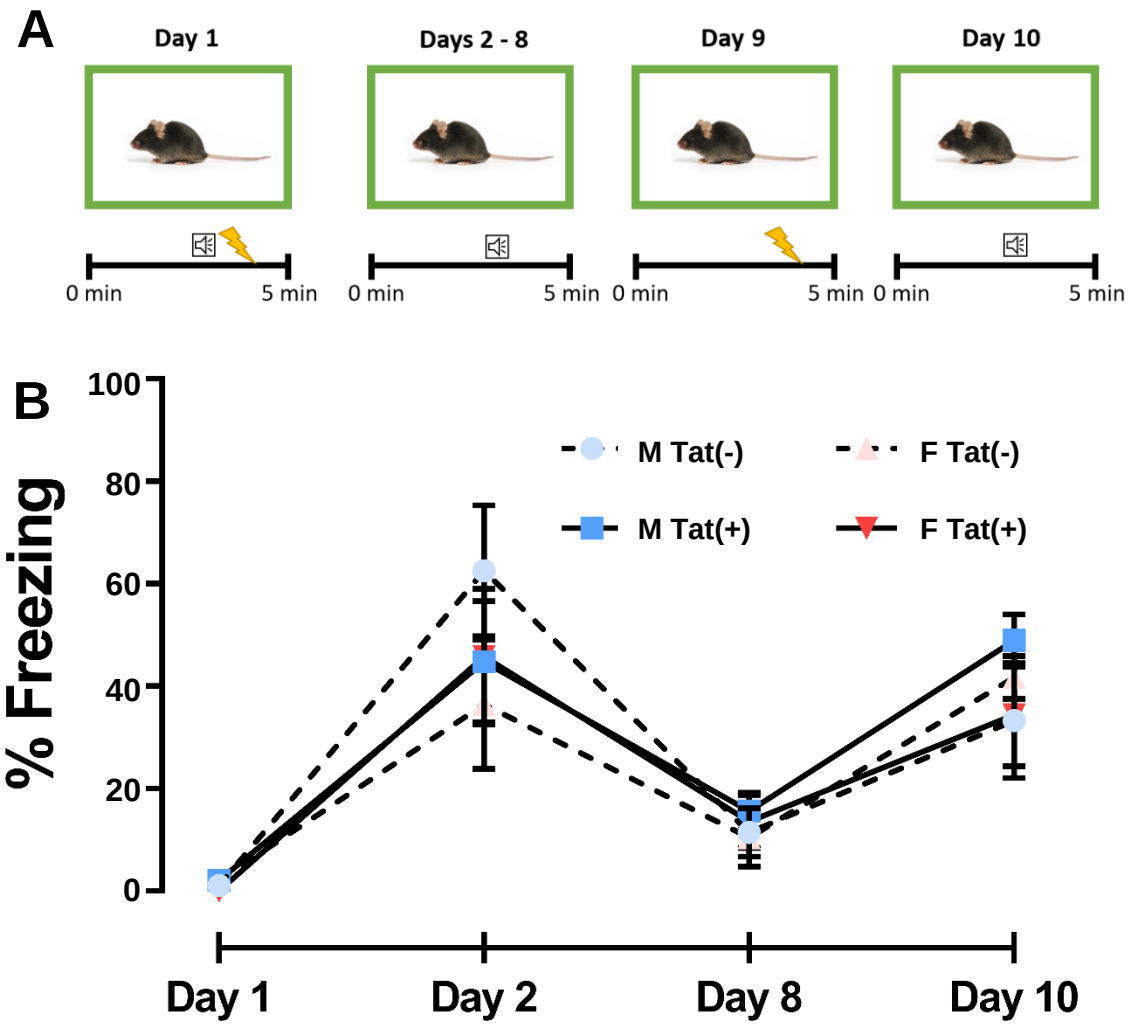

Figure 8. Reinstatement: All subjects show typical acquisition, extinction and reinstatement effects. **(A)** Acquisition and extinction proceeded as previously described in **Figure 5**. On Day 9, subjects underwent reinstatement treatment in which the US is presented independent of and CSs. Notably this does not represent reacquisition (CS→US), but is instead a relapse in CR solely due to the unsignaled US presentation. On Day 10, subjects underwent testing in which the CS was presented independent of the US. **(B)** Data from these experiments show normal acquisition, extinction, and reinstatement (all $p > .05$), confirming the hypothesis that reinstatement would be unaffected by Tat. Further, this data shows that the failure to show relapse in the renewal experiments was not due to a failure to recall acquisition memories following extinction. On Day 10, the increase in CR intensity to the CS would not be possible if the subjects did not maintain acquisition memories (CS→US).

REFERENCES

1. Bouton, M.E. and R.C. Bolles, *Role of conditioned contextual stimuli in reinstatement of extinguished fear.* J Exp Psychol Anim Behav Process, 1979. **5**(4): p. 368-78.

2. Bouton, M.E. and D.A. King, *Contextual control of the extinction of conditioned fear: Tests for the associative value of the context.* Journal of Experimental Psychology: Animal Behavior Processes, 1983. **9**: p. 248-265.

3. Chen, W., et al., *Neural circuits involved in the renewal of extinguished fear.* IUBMB Life, 2017. **69**(7): p. 470-478.

4. Corcoran, K.A. and S. Maren, *Hippocampal inactivation disrupts contextual retrieval of fear memory after extinction.* Journal of Neuroscience, 2001. **21**(5): p. 1720-1726.

5. Harris, J.A., et al., *Contextual control over conditioned responding in an extinction paradigm.* J Exp Psychol Anim Behav Process, 2000. **26**(2): p. 174-85.

6. Sharpe, M. and S. Killcross, *The prelimbic cortex uses contextual cues to modulate responding towards predictive stimuli during fear renewal.* Neurobiol Learn Mem, 2015. **118**: p. 20-9.

7. Cabana-Domínguez, J., et al., *Reduced cue-induced reinstatement of cocaine-seeking behavior in Plcb1 +/− mice.* Translational Psychiatry, 2021. **11**(1): p. 521.

8. Everitt, B.J. and T.W. Robbins, *From the ventral to the dorsal striatum: devolving views of their roles in drug addiction.* Neuroscience & Biobehavioral Reviews, 2013. **37**(9): p. 1946-1954.

9. Hitora-Imamura, N., et al., *Prefrontal dopamine regulates fear reinstatement through the downregulation of extinction circuits.* eLife, 2015. **4**: p. e08274.

10. Lammel, S., et al., *Input-specific control of reward and aversion in the ventral tegmental area.* Nature, 2012. **491**(7423): p. 212-217.

11. Rogers, J.L., R.E. Ghee S Fau - See, and R.E. See, *The neural circuitry underlying reinstatement of heroin-seeking behavior in an animal model of relapse.* (0306-4522 (Print)).

12. Sierra-Mercado Jr, D., et al., *Inactivation of the ventromedial prefrontal cortex reduces expression of conditioned fear and impairs subsequent recall of extinction.* European Journal of Neuroscience, 2006. **24**(6): p. 1751-1758.

13. Burgos-Robles, A., et al., *Consolidation of fear extinction requires NMDA receptor-dependent bursting in the ventromedial prefrontal cortex.* Neuron, 2007. **53**(6): p. 871-880.

14. Mueller, D., J.T. Porter, and G.J. Quirk, *Noradrenergic signaling in infralimbic cortex increases cell excitability and strengthens memory for fear extinction.* J Neurosci, 2008. **28**(2): p. 369-75.

15. Chhatwal, J.P., et al., *Regulation of gephyrin and GABAA receptor binding within the amygdala after fear acquisition and extinction.* Journal of Neuroscience, 2005. **25**(2): p. 502-506.

16. McDonald, A.J., *Cortical pathways to the mammalian amygdala.* Progress in neurobiology, 1998. **55**(3): p. 257-332.

17. Ehrlich, I., et al., *Amygdala inhibitory circuits and the control of fear memory.* Neuron, 2009. **62**(6): p. 757-71.

18. Milad, M.R. and G.J. Quirk, *Neurons in medial prefrontal cortex signal memory for fear extinction.* Nature, 2002. **420**(6911): p. 70-74.

19. Cassell, M.D., L.J. Freedman, and C. Shi, *The intrinsic organization of the central extended amygdala.* Annals of the New York Academy of Sciences, 1999. **877**(1): p. 217-241.

20. Kim, W.B. and J.-H. Cho, *Encoding of contextual fear memory in hippocampal–amygdala circuit.* Nature Communications, 2020. **11**(1): p. 1382.

21. Bouton, M.E., *Context, ambiguity, and unlearning: sources of relapse after behavioral extinction.* Biological Psychiatry, 2002. **52**(10): p. 976-986.

22. Cammarota, M., et al., *Retrieval and the extinction of memory.* Cellular and molecular neurobiology, 2005. **25**(3): p. 465-474.

23. Orsini, C.A., et al., *Hippocampal and prefrontal projections to the basal amygdala mediate contextual regulation of fear after extinction.* Journal of Neuroscience, 2011. **31**(47): p. 17269-17277.

24. Corcoran, K.A., et al., *Hippocampal inactivation disrupts the acquisition and contextual encoding of fear extinction.* Journal of Neuroscience, 2005. **25**(39): p. 8978-8987.

25. Gartner, S., *HIV infection and dementia.* Science, 2000. **287**(5453): p. 602-4.

26. Kaul, M., G.A. Garden, and S.A. Lipton, *Pathways to neuronal injury and apoptosis in HIV-associated dementia.* Nature, 2001. **410**(6831): p. 988-94.

27. Ellis, R., D. Langford, and E. Masliah, *HIV and antiretroviral therapy in the brain: neuronal injury and repair.* Nat Rev Neurosci, 2007. **8**(1): p. 33-44.

28. Harrison, K.M., R. Song, and X. Zhang, *Life expectancy after HIV diagnosis based on national HIV surveillance data from 25 states, United States.* J Acquir Immune Defic Syndr, 2010. **53**(1): p. 124-30.

29. Fiala, M., et al., *TNF-alpha opens a paracellular route for HIV-1 invasion across the blood-brain barrier.* Mol Med, 1997. **3**(8): p. 553-64.

30. Kalams, S.A. and B.D. Walker, *Cytotoxic T lymphocytes and HIV-1 related neurologic disorders.* Curr Top Microbiol Immunol, 1995. **202**: p. 79-88.

31. Sasseville, V.G., et al., *Monocyte adhesion to endothelium in simian immunodeficiency virus-induced AIDS encephalitis is mediated by vascular cell adhesion molecule-1/alpha 4 beta 1 integrin interactions.* Am J Pathol, 1994. **144**(1): p. 27-40.

32. Antinori, A., et al., *Updated research nosology for HIV-associated neurocognitive disorders.* Neurology, 2007. **69**(18): p. 1789-99.

33. Heaton, R.K., et al., *HIV-associated neurocognitive disorders before and during the era of combination antiretroviral therapy: differences in rates, nature, and predictors.* Journal of neurovirology, 2011. **17**(1): p. 3-16.

34. Vago, L., et al., *Pathological findings in the central nervous system of AIDS patients on assumed antiretroviral therapeutic regimens: retrospective study of 1597 autopsies.* Aids, 2002. **16**(14): p. 1925-1928.

35. Smail, R.C. and B.J. Brew, *HIV-associated neurocognitive disorder.* Handbook of clinical neurology, 2018. **152**: p. 75-97.

36. Saylor, D., et al., *HIV-associated neurocognitive disorder—pathogenesis and prospects for treatment.* Nature Reviews Neurology, 2016. **12**(4): p. 234-248.

37. Gelman, B.B., *Neuropathology of HAND with suppressive antiretroviral therapy: encephalitis and neurodegeneration reconsidered.* Current Hiv/Aids Reports, 2015. **12**(2): p. 272-279.

38. Ferrington, D.A. and D.S. Gregerson, *Immunoproteasomes: structure, function, and antigen presentation.* Progress in molecular biology and translational science, 2012. **109**: p. 75-112.

39. Li, L., et al., *Roles of HIV-1 auxiliary proteins in viral pathogenesis and host-pathogen interactions.* Cell Research, 2005. **15**(11): p. 923-934.

40. Frankel, A.D. and J.A. Young, *HIV-1: fifteen proteins and an RNA.* (0066-4154 (Print)).

41. Everall, I.P., et al., *Cortical synaptic density is reduced in mild to moderate human immunodeficiency virus neurocognitive disorder. HNRC Group. HIV Neurobehavioral Research Center.* Brain Pathol, 1999. **9**(2): p. 209-17.

42. Masliah, E., et al., *Dendritic injury is a pathological substrate for human immunodeficiency virus-related cognitive disorders. HNRC Group. The HIV Neurobehavioral Research Center.* Ann Neurol, 1997. **42**(6): p. 963-72.

43. Fitting, S., et al., *Interactive HIV-1 Tat and morphine-induced synaptodendritic injury is triggered through focal disruptions in Na(+) influx, mitochondrial instability, and Ca(2)(+) overload.* J Neurosci, 2014. **34**(38): p. 12850-64.

44. Green, M.V., et al., *Scaling Synapses in the Presence of HIV.* Neurochem Res, 2018.

45. Haughey, N.J., et al., *Involvement of inositol 1,4,5-trisphosphate-regulated stores of intracellular calcium in calcium dysregulation and neuron cell death caused by HIV-1 protein tat.* J Neurochem, 1999. **73**(4): p. 1363-74.

46. Mattson, M.P., N.J. Haughey, and A. Nath, *Cell death in HIV dementia.* Cell Death Differ, 2005. **12 Suppl 1**: p. 893-904.

47. Chen, P., et al., *The Tat protein of HIV-1 induces tumor necrosis factor-alpha production. Implications for HIV-1-associated neurological diseases.* J Biol Chem, 1997. **272**(36): p. 22385-8.

48. Cheng, J., et al., *Neuronal excitatory properties of human immunodeficiency virus type 1 Tat protein.* Neuroscience, 1998. **82**(1): p. 97-106.

49. El-Hage, N., et al., *Morphine exacerbates HIV-1 Tat-induced cytokine production in astrocytes through convergent effects on $[Ca^{2+}]_i$, NF-κB trafficking and transcription.* PLoS One, 2008. **3**(12): p. e4093.

50. El-Hage, N., et al., *Synergistic increases in intracellular Ca^{2+}, and the release of MCP-1, RANTES, and IL-6 by astrocytes treated with opiates and HIV-1 Tat.* Glia, 2005. **50**(2): p. 91-106.

51. Kutsch, O., et al., *Induction of the chemokines interleukin-8 and IP-10 by human immunodeficiency virus type 1 tat in astrocytes.* J Virol, 2000. **74**(19): p. 9214-21.

52. Lipton, S.A., *Human immunodeficiency virus-infected macrophages, gp120, and N-methyl-D-aspartate receptor-mediated neurotoxicity.* Ann Neurol, 1993. **33**(2): p. 227-8.

53. Liu, Q., et al., *Signaling pathways from cannabinoid receptor-1 activation to inhibition of N-methyl-D-aspartic acid mediated calcium influx and neurotoxicity in dorsal root ganglion neurons.* J Pharmacol Exp Ther, 2009. **331**(3): p. 1062-70.

54. Magnuson, D.S., et al., *Human immunodeficiency virus type 1 tat activates non-N-methyl-D-aspartate excitatory amino acid receptors and causes neurotoxicity.* Ann Neurol, 1995. **37**(3): p. 373-80.

55. Nath, A., *Human immunodeficiency virus (HIV) proteins in neuropathogenesis of HIV dementia.* J Infect Dis, 2002. **186 Suppl 2**: p. S193-8.

56. Eugenin, E.A., et al., *HIV-tat induces formation of an LRP-PSD-95- NMDAR-nNOS complex that promotes apoptosis in neurons and astrocytes.* Proc Natl Acad Sci U S A, 2007. **104**(9): p. 3438-43.

57. Longordo, F., et al., *The human immunodeficiency virus-1 protein transactivator of transcription up-regulates N-methyl-D-aspartate receptor function by acting at metabotropic glutamate receptor 1 receptors coexisting on human and rat brain noradrenergic neurones.* J Pharmacol Exp Ther, 2006. **317**(3): p. 1097-105.

58. Raybuck, J.D., N.J. Hargus, and S.A. Thayer, *A GluN2B-Selective NMDAR Antagonist Reverses Synapse Loss and Cognitive Impairment Produced by the HIV-1 Protein Tat.* J Neurosci, 2017. **37**(33): p. 7837-7847.

59. Haughey, N.J., et al., *HIV-1 Tat through phosphorylation of NMDA receptors potentiates glutamate excitotoxicity.* Journal of Neurochemistry, 2001. **78**(3): p. 457-467.

60. Connolly, C.G., et al., *Altered functional response to risky choice in HIV infection.* PLoS One, 2014. **9**(10): p. e111583.

61. Cysique, L.A., P. Maruff, and B.J. Brew, *Prevalence and pattern of neuropsychological impairment in human immunodeficiency virus-infected/acquired immunodeficiency syndrome (HIV/AIDS) patients across pre- and post-highly active antiretroviral therapy eras: a combined study of two cohorts.* J Neurovirol, 2004. **10**(6): p. 350-7.

62. Ernst, T., L. Chang, and S. Arnold, *Increased glial metabolites predict increased working memory network activation in HIV brain injury.* Neuroimage, 2003. **19**(4): p. 1686-93.

63. Garvey, L.J., D. Yerrakalva, and A. Winston, *Correlations between Computerized Battery Testing and a Memory Questionnaire for Identification of Neurocognitive Impairment in HIV Type 1-Infected Subjects on Stable Antiretroviral Therapy.* AIDS Research and Human Retroviruses, 2008. **25**(8): p. 765-769.

64. Jacobs, I.R., et al., *Inhibitory Control Deficits Associated with Upregulation of CB1R in the HIV-1 Tat Transgenic Mouse Model of Hand.* J Neuroimmune Pharmacol, 2019.

65. Wang, Y.Q., et al., *Selective impairments of alerting and executive control in HIV-infected patients: evidence from attention network test.* Behav Brain Funct, 2017. **13**(1): p. 11.

66. Xu, C., et al., *Inhibitory Neurotransmission Is Sex-Dependently Affected by Tat Expression in Transgenic Mice and Suppressed by the Fatty Acid Amide Hydrolase Enzyme Inhibitor PF3845 via Cannabinoid Type-1 Receptor Mechanisms. LID - 10.3390/cells11050857 [doi] LID - 857.* (2073-4409 (Electronic)).

67. Munakata, Y., et al., *A unified framework for inhibitory control.* Trends in cognitive sciences, 2011. **15**(10): p. 453-459.

68. League, A.F., et al., *Monoacylglycerol Lipase Inhibitor MJN110 Reduces Neuronal Hyperexcitability, Restores Dendritic Arborization Complexity, and Regulates Reward-Related Behavior in Presence of HIV-1 Tat.* Frontiers in Neurology, 2021. **12**.

69. Xu, C., et al., *Endocannabinoids exert CB(1) receptor-mediated neuroprotective effects in models of neuronal damage induced by HIV-1 Tat protein.* (1095-9327 (Electronic)).

70. Carey, A.N., et al., *Expression of HIV-Tat protein is associated with learning and memory deficits in the mouse.* Behav Brain Res, 2012. **229**(1): p. 48-56.

71. Fitting, S., et al., *Synaptic dysfunction in the hippocampus accompanies learning and memory deficits in human immunodeficiency virus type-1 Tat transgenic mice.* Biol Psychiatry, 2013. **73**(5): p. 443-53.

72. Hahn, Y.K., et al., *Central HIV-1 Tat exposure elevates anxiety and fear conditioned responses of male mice concurrent with altered mu-opioid receptor-mediated G-protein activation and beta-arrestin 2 activity in the forebrain.* Neurobiol Dis, 2016. **92**(Pt B): p. 124-36.

73. Kesby, J.P., et al., *Effects of HIV/TAT protein expression and chronic selegiline treatment on spatial memory, reversal learning and neurotransmitter levels in mice.* Behav Brain Res, 2016. **311**: p. 131-140.

74. Marks, W.D., et al., *HIV-1 Tat causes cognitive deficits and selective loss of parvalbumin, somatostatin, and neuronal nitric oxide synthase expressing hippocampal CA1 interneuron subpopulations.* J Neurovirol, 2016. **22**(6): p. 747-762.

75. Nookala, A.R., et al., *Methamphetamine augment HIV-1 Tat mediated memory deficits by altering the expression of synaptic proteins and neurotrophic factors.* Brain Behav Immun, 2018. **71**: p. 37-51.

76. Marks, W.A.-O., et al., *HIV-1 Tat and Morphine Differentially Disrupt Pyramidal Cell Structure and Function and Spatial Learning in Hippocampal Area CA1: Continuous versus Interrupted Morphine Exposure. LID - ENEURO.0547-20.2021 [pii] LID - 10.1523/ENEURO.0547-20.2021 [doi].* (2373-2822 (Electronic)).

77. McLane, V.D., et al., *HIV-1 Tat reduces apical dendritic spine density throughout the trisynaptic pathway in the hippocampus of male transgenic mice.* (1872-7972 (Electronic)).

78. Kesby, J.P., A. Markou, and S. Semenova, *The effects of HIV-1 regulatory TAT protein expression on brain reward function, response to psychostimulants and delay-dependent memory in mice.* Neuropharmacology, 2016. **109**: p. 205-215.

79. Nass, S.R., et al., *Neurodegeneration Within the Amygdala Is Differentially Induced by Opioid and HIV-1 Tat Exposure.* Frontiers in Neuroscience, 2022. **16**.

80. Nass, S.R., et al., *HIV-1 Tat and morphine decrease murine inter-male social interactions and associated oxytocin levels in the prefrontal cortex, amygdala, and hypothalamic paraventricular nucleus.* Horm Behav, 2021. **133**: p. 105008.

81. Brandão, M.L., et al., *Different patterns of freezing behavior organized in the periaqueductal gray of rats: association with different types of anxiety.* (0166-4328 (Print)).

82. Rescorla, R.A. and A. Wagner, *A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and nonreinforcement*. 1972.

83. Pavlov, I.P., *Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex*. Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex. 1927, Oxford, England: Oxford Univ. Press. xv, 430-xv, 430.

84. Davis, M., *The role of the amygdala in conditioned fear.* 1992.

85. Fanselow, M.S. and A.M. Poulos, *The neuroscience of mammalian associative learning.* Annual review of psychology, 2005. **56**(1): p. 207-234.

86. LeDoux, J.E., *Emotion circuits in the brain.* 2000.

87. Maren, S., *Neurobiology of Pavlovian fear conditioning.* Annual review of neuroscience, 2001. **24**(1): p. 897-931.

88. Samson, R.D. and D. Paré, *Activity-dependent synaptic plasticity in the central nucleus of the amygdala.* Journal of Neuroscience, 2005. **25**(7): p. 1847-1855.

89. Millhouse, O.E., *The intercalated cells of the amygdala.* Journal of Comparative Neurology, 1986. **247**(2): p. 246-271.

90. Passey, G.E., *The influence of intensity of unconditioned stimulus upon acquisition of a conditioned response.* Journal of Experimental Psychology, 1948. **38**: p. 420-428.

91. Chan, C.K.J. and J.A. Harris, *Extinction of Pavlovian conditioning: The influence of trial number and reinforcement history.* Behav Processes, 2017. **141**(Pt 1): p. 19-25.

92. Bruce-Keller, A.J., et al., *Morphine causes rapid increases in glial activation and neuronal injury in the striatum of inducible HIV-1 Tat transgenic mice.* Glia, 2008. **56**(13): p. 1414-27.

93. Hahn, Y.K., et al., *Effects of chronic HIV-1 Tat exposure in the CNS: heightened vulnerability of males versus females to changes in cell numbers, synaptic integrity, and behavior.* Brain Struct Funct, 2015. **220**(2): p. 605-23.

94. Curzon, P., N.R. Rustay, and K.E. Browman, *Cued and Contextual Fear Conditioning for Rodents*, in *Methods of Behavior Analysis in Neuroscience*, nd and J.J. Buccafusco, Editors. 2009: Boca Raton (FL).

95. Myers, K.M. and M. Davis, *Mechanisms of fear extinction.* Mol Psychiatry, 2007. **12**(2): p. 120-50.

96. Tipps, M.E., et al., *Delay and trace fear conditioning in C57BL/6 and DBA/2 mice: issues of measurement and performance.* Learn Mem, 2014. **21**(8): p. 380-93.

97. Lattal, K.M. and D.K. Maughan, *A parametric analysis of factors affecting acquisition and extinction of contextual fear in C57BL/6 and DBA/2 mice.* Behav Processes, 2012. **90**(1): p. 49-57.

98. Nguyen, P.V., et al., *Strain-dependent differences in LTP and hippocampus-dependent memory in inbred mice.* Learn Mem, 2000. **7**(3): p. 170-9.

99. Lenselink, A.M., et al., *Strain Differences in Presynaptic Function: PROTEOMICS, ULTRASTRUCTURE, AND PHYSIOLOGY OF HIPPOCAMPAL SYNAPSES IN DBA/2J AND C57Bl/6J MICE.* J Biol Chem, 2015. **290**(25): p. 15635-15645.

100. Pick, C.G. and J. Yanai, *Studies into the mechanisms of strain differences in hippocampus-related behaviors.* Behavior Genetics, 1989. **19**(2): p. 315-325.

101. Patacchioli, F.R., et al., *Strain-dependent differences in hippocampal glucocorticoid binding capacity and active avoidance in the mouse.* Behav Brain Res, 1990. **37**(2): p. 185-8.

102. Nesil, T., et al., *Nicotine attenuates the effect of HIV-1 proteins on the neural circuits of working and contextual memories.* Molecular Brain, 2015. **8**(1): p. 43.

103. Nass, S.R., et al., *HIV-1 Tat and morphine decrease murine inter-male social interactions and associated oxytocin levels in the prefrontal cortex, amygdala, and hypothalamic paraventricular nucleus.* (1095-6867 (Electronic)).

104. Dalm, S., et al., *Age-related changes in hypothalamic-pituitary-adrenal axis activity of male C57BL/6J mice.* Neuroendocrinology, 2005. **81**(6): p. 372-80.

105. Peyrot, C., et al., *A review on how stress modulates fear conditioning: Let's not forget the role of sex and sex hormones.* Behaviour Research and Therapy, 2020. **129**: p. 103615.

106. Merz, C.J., et al., *Stress differentially affects fear conditioning in men and women.* Psychoneuroendocrinology, 2013. **38**(11): p. 2529-2541.

107. Zorawski, M., et al., *Effects of stress and sex on acquisition and consolidation of human fear conditioning.* Learning & memory, 2006. **13**(4): p. 441-450.

108. Stroud, L.R., P. Salovey, and E.S. Epel, *Sex differences in stress responses: social rejection versus achievement stress.* Biological psychiatry, 2002. **52**(4): p. 318-327.

109. Salahuddin, M.F., F. Mahdi, and J.J. Paris, *HIV-1 Tat Dysregulates the Hypothalamic-Pituitary-Adrenal Stress Axis and Potentiates Oxycodone-Mediated Psychomotor and Anxiety-Like Behavior of Male Mice.* Int J Mol Sci, 2020. **21**(21).

110. Lerch, S., et al., *The scent of stress: environmental challenge in the peripartum environment of mice affects emotional behaviours of the adult offspring in a sex-specific manner.* Lab Anim, 2016. **50**(3): p. 167-78.

111. Quirk, G.J. and D. Mueller, *Neural Mechanisms of Extinction Learning and Retrieval.* Neuropsychopharmacology, 2008. **33**(1): p. 56-72.

112. Nader, K., et al., *Damage to the lateral and central, but not other, amygdaloid nuclei prevents the acquisition of auditory fear conditioning.* Learning & Memory, 2001. **8**(3): p. 156-163.

113. Anglada-Figueroa, D. and G.J. Quirk, *Lesions of the basal amygdala block expression of conditioned fear but not extinction.* Journal of Neuroscience, 2005. **25**(42): p. 9680-9685.

114. Akirav, I., H. Raizel, and M. Maroun, *Enhancement of conditioned fear extinction by infusion of the GABAA agonist muscimol into the rat prefrontal cortex and amygdala.* European Journal of Neuroscience, 2006. **23**(3): p. 758-764.

115. Berlau, D.J. and J.L. McGaugh, *Enhancement of extinction memory consolidation: the role of the noradrenergic and GABAergic systems within the basolateral amygdala.* Neurobiology of learning and memory, 2006. **86**(2): p. 123-132.

116. Sotres-Bayon, F., D.E.A. Bush, and J.E. LeDoux, *Acquisition of fear extinction requires activation of NR2B-containing NMDA receptors in the lateral amygdala.* Neuropsychopharmacology, 2007. **32**(9): p. 1929-1940.

117. Waddell, J., C. Dunnett, and W.A. Falls, *C57BL/6J and DBA/2J mice differ in extinction and renewal of extinguished conditioned fear.* Behav Brain Res, 2004. **154**(2): p. 567-76.

118. Paris, J.J., et al., *Effects of conditional central expression of HIV-1 tat protein to potentiate cocaine-mediated psychostimulation and reward among male mice.* (1740-634X (Electronic)).

119. Colon, L., et al., *Sexual differentiation of contextual fear responses.* Learn Mem, 2018. **25**(5): p. 230-240.

120. Colon, L.M. and A.M. Poulos, *Contextual processing elicits sex differences in dorsal hippocampus activation following footshock and context fear retrieval.* Behav Brain Res, 2020. **393**: p. 112771.

121. Asok, A., et al., *Sex Differences in Remote Contextual Fear Generalization in Mice.* Front Behav Neurosci, 2019. **13**: p. 56.

122. Baran, S.E., et al., *Prefrontal cortex lesions and sex differences in fear extinction and perseveration.* Learn Mem, 2010. **17**(5): p. 267-78.

123. Baran, S.E., et al., *Chronic stress and sex differences on the recall of fear conditioning and extinction.* Neurobiol Learn Mem, 2009. **91**(3): p. 323-32.

124. Gruene, T.M., et al., *Sexually divergent expression of active and passive conditioned fear responses in rats.* Elife, 2015. **4**.

125. Voulo, M.E. and R.G. Parsons, *Response-specific sex difference in the retention of fear extinction.* Learn Mem, 2017. **24**(6): p. 245-251.

126. Balogh, S.A. and J.M. Wehner, *Inbred mouse strain differences in the establishment of long-term fear memory.* Behav Brain Res, 2003. **140**(1-2): p. 97-106.

127. Boynton, F.D.D., et al., *Doxycycline induces dysbiosis in female C57BL/6NCrl mice.* BMC Res Notes, 2017. **10**(1): p. 644.

128. Chu, C., et al., *The microbiota regulate neuronal function and fear extinction learning.* Nature, 2019. **574**(7779): p. 543-548.

129. Tang, W., et al., *Roles of Gut Microbiota in the Regulation of Hippocampal Plasticity, Inflammation, and Hippocampus-Dependent Behaviors.* Front Cell Infect Microbiol, 2020. **10**: p. 611014.

130. Li, Y., et al., *Activation of astrocytes in hippocampus decreases fear memory through adenosine A1 receptors.* Elife, 2020. **9**.

131. Shelkar, G.P., J. Liu, and S.M. Dravid, *Astrocytic NMDA Receptors in the Basolateral Amygdala Contribute to Facilitation of Fear Extinction.* Int J Neuropsychopharmacol, 2021. **24**(11): p. 907-919.

132. Dickens, A.M., et al., *Chronic low-level expression of HIV-1 Tat promotes a neurodegenerative phenotype with aging.* Scientific Reports, 2017. **7**(1): p. 7748.

133. Bray, N., *Calculating error.* Nature Reviews Neuroscience, 2016. **17**(11): p. 670-670.

134. Hikosaka, O., *The habenula: from stress evasion to value-based decision-making.* Nature reviews neuroscience, 2010. **11**(7): p. 503-513.

135. Hermes, D.J., et al., *Escalating morphine dosing in HIV-1 Tat transgenic mice with sustained Tat exposure reveals an allostatic shift in neuroinflammatory regulation accompanied by increased neuroprotective non-endocannabinoid lipid signaling molecules and amino acids.* J Neuroinflammation, 2020. **17**(1): p. 345.

136. Carey, A.N., et al., *Expression of HIV-Tat protein is associated with learning and memory deficits in the mouse.* Behavioural Brain Research, 2012. **229**(1): p. 48-56.

137. Dong, Z., et al., *Hippocampal long-term depression mediates spatial reversal learning in the Morris water maze.* Neuropharmacology, 2013. **64**: p. 65-73.

138. Joshi, C.R., et al., *Astrocyte HIV-1 Tat Differentially Modulates Behavior and Brain MMP/TIMP Balance During Short and Prolonged Induction in Transgenic Mice.* Front Neurol, 2020. **11**: p. 593188.

139. Paris, J.J., et al., *Anxiety-like behavior of mice produced by conditional central expression of the HIV-1 regulatory protein, Tat.* Psychopharmacology (Berl), 2014. **231**(11): p. 2349-60.

140. Krishef, C.H., *Fundamental approaches to single subject design and analysis*. 1991, Melbourne, FL, US: Robert E Krieger Publishing Co. xi, 142-xi, 142.

141. Satake, E., V. Jagaroo, and D.L. Maxwell, *Handbook of statistical methods: Single subject design*. 2008: Plural Publishing.

142. Gruene, T.M., et al., *Sexually divergent expression of active and passive conditioned fear responses in rats.* eLife, 2015. **4**: p. e11352.

143. Trott, J.M., et al., *Conditional Freezing, Flight and Darting?* bioRxiv, 2021: p. 2021.12.02.470975.

144. Fanselow, M.S. and L.S. Lester, *A functional behavioristic approach to aversively motivated behavior: predatory imminence as a determinant of the topography of defensive behavior.* 1988.

145. Fadok, J.P., et al., *A competitive inhibitory circuit for selection of active and passive fear responses.* Nature, 2017. **542**(7639): p. 96-100.

146. Hersman, S., et al., *Stimulus salience determines defensive behaviors elicited by aversively conditioned serial compound auditory stimuli.* Elife, 2020. **9**: p. e53803.

147. Bouton, M.E., S. Mineka, and D.H. Barlow, *A modern learning theory perspective on the etiology of panic disorder.* Psychological review, 2001. **108**(1): p. 4.

148. De Franceschi, G., et al., *Vision Guides Selection of Freeze or Flight Defense Strategies in Mice.* Curr Biol, 2016. **26**(16): p. 2150-4.

149. Heinemans, M. and M.A. Moita, *Looming stimuli reliably drive innate, but not learned, defensive responses in rats.* bioRxiv, 2022: p. 2022.02.07.479432.

150. Takahashi, L.K., *Olfactory systems and neural circuits that modulate predator odor fear. Front Behav Neurosci. 2014; 8: 72*. 2014.

151. Blanchard, R.J., et al., *Cue and context conditioning of defensive behaviors to cat odor stimuli.* Neuroscience & Biobehavioral Reviews, 2001. **25**(7-8): p. 587-595.

152. Hargus, N.J. and S.A. Thayer, *Human immunodeficiency virus-1 Tat protein increases the number of inhibitory synapses between hippocampal neurons in culture.* J Neurosci, 2013. **33**(45): p. 17908-20.

153. Nass, S.R., et al., *Chronic HIV-1 Tat exposure alters anterior cingulate cortico-basal ganglia-thalamocortical synaptic circuitry, associated behavioral control, and immune regulation in male mice.* Brain Behav Immun Health, 2020. **5**.

154. Kim, H.J., K.A. Martemyanov, and S.A. Thayer, *Human immunodeficiency virus protein Tat induces synapse loss via a reversible process that is distinct from cell death.* J Neurosci, 2008. **28**(48): p. 12604-13.

155. Fitting, S., et al., *Interactive comorbidity between opioid drug abuse and HIV-1 Tat: chronic exposure augments spine loss and sublethal dendritic pathology in striatal neurons.* (1525-2191 (Electronic)).

156. McLane, V.D., et al., *HIV-1 Tat reduces apical dendritic spine density throughout the trisynaptic pathway in the hippocampus of male transgenic mice.* Neuroscience Letters, 2022. **782**: p. 136688.

157. Schier, C.J., et al., *Selective Vulnerability of Striatal D2 versus D1 Dopamine Receptor-Expressing Medium Spiny Neurons in HIV-1 Tat Transgenic Male Mice.* The Journal of Neuroscience, 2017. **37**(23): p. 5758.

158. Pilati, N., et al., *A rapid method combining Golgi and Nissl staining to study neuronal morphology and cytoarchitecture.* Journal of Histochemistry & Cytochemistry, 2008. **56**(6): p. 539-550.

159. Van Spronsen, M. and C.C. Hoogenraad, *Synapse pathology in psychiatric and neurologic disease.* Current neurology and neuroscience reports, 2010. **10**(3): p. 207-214.

160. McLeod, F., et al., *Evaluation of synapse density in hippocampal rodent brain slices.* JoVE (Journal of Visualized Experiments), 2017(128): p. e56153.

161. Prange, O., et al., *A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin.* Proceedings of the National Academy of Sciences, 2004. **101**(38): p. 13915-13920.

162. Markram, H., et al., *Interneurons of the neocortical inhibitory system.* Nature reviews neuroscience, 2004. **5**(10): p. 793-807.

163. Georgiou, C., et al., *A subpopulation of cortical VIP-expressing interneurons with highly dynamic spines.* (2399-3642 (Electronic)).

164. Xu, C., et al., *Cannabinoids Occlude the HIV-1 Tat-Induced Decrease in GABAergic Neurotransmission in Prefrontal Cortex Slices.* (1557-1904 (Electronic)).

165. Cirino, T.A.-O., et al., *Region-specific effects of HIV-1 Tat on intrinsic electrophysiological properties of pyramidal neurons in mouse prefrontal cortex and hippocampus.* (1522-1598 (Electronic)).

166. Marks, W.D., et al., *HIV-1 Tat and Morphine Differentially Disrupt Pyramidal Cell Structure and Function and Spatial Learning in Hippocampal Area CA1: Continuous versus Interrupted Morphine Exposure.* eneuro, 2021. **8**(3): p. ENEURO.0547-20.2021.